การโคลนนิ่งและการศึกษาลำดับเบสของยีน 3ABC ของเชื้อไวรัสโรคปากและเท้าเปื่อย ไทป์โอ ของสุกรที่แยกได้จากจังหวัดราชบุรีในปีพ.ศ. 2548

นางสาวกุลิสรา มรุปัณฑ์ธร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CLONING AND SEQUENCING OF 3ABC GENE OF FOOT AND MOUTH DISEASE VIRUS TYPE O FROM A THAI PIG ISOLATE FROM RATCHABURI PROVINCE IN 2005

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Medicine
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โรคปากและเท้าเปื่อย (Foot and mouth disease; FMD) ในสุกรเป็นโรคระบาดสัตว์ที่สำคัญของประเทศไทย ที่มีความรุนแรงและมีการระบาดสูง นอกจากจะส่งผลกระทบต่อผลผลิตและผลิตภัณฑ์จากสุกรแล้ว ยังก่อให้เกิดการกีดกันทางการค้าระหว่างประเทศ ส่งผลให้เกิดความเสียหายต่อธุรกิจการเลี้ยงสุกรเป็นอย่างมาก เชื้อไวรัสปากและเท้าเปื่อยที่พบมากที่สุด และมีการระบาดล่าสุดในปี พ.ศ. 2548 คือ ไทป์โอ การตรวจคัดแยกสุกรที่ติดเชื้อไวรัสชนิดนี้โดยธรรมชาติออกจากสุกรที่ได้รับวัคซีนเป็นสิ่งจำเป็นในการควบคุมการระบาดของโรคนี้ การศึกษาครั้งนี้จึงทำการสกัดแยกอาร์เอ็นเอของเชื้อไวรัสโรคปากและเท้าเปื่อยไทป์โอ ที่ได้จากสุกรป่วยในจังหวัดราชบุรีจากตุ่มน้ำและเยื่อเมือกที่ลอกหลุดบริเวณไรกีบของสุกร ทำการสังเคราะห์สาย cDNA ด้วยเทคนิค reverse transcription และตรวจยืนยันไทป์โอของเชื้อไวรัส โดยการเพิ่มปริมาณสารพันธุกรรมของยีน O-1C_{124} ด้วยเทคนิค PCR และทำการเพิ่มจำนวนสารพันธุกรรมส่วนยีน 3ABC ทั้งสายของเชื้อไวรัสด้วยเทคนิค PCR นำ PCR products ที่ได้ไปโคลนเข้าสู่เวคเตอร์ pET160/GW/TOPO® แล้วทำการวิเคราะห์ และเปรียบเทียบลำดับนิวคลีโอไทด์ของยีน 3ABC กับลำดับนิวคลีโอไทด์ที่มีรายงานอยู่ใน Genbank พบว่า ได้โคลนที่มียีน 3ABC อยู่ภายใน expression vector ซึ่งแยกได้จากสุกรในจังหวัดราชบุรี จำนวน 5 โคลน โดยมีขนาด 1,303 bp และผลจากการวิเคราะห์ด้วย phylogenetic tree พบว่า ลำดับนิวคลีโอไทด์ที่ได้เหมือนกับเชื้อไวรัสโรคปากและเท้าเปื่อย ไทป์โอ สเตรน Tibet/CHA/99 100%

ภาควิชา อายุรศาสตร์ ลายมือชื่อนิสิต กุลิสรา มรุปัณฑ์ธร

สาขาวิชา อายุรศาสตร์สัตวแพทย์ ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก

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KULISARA MARUPANTHORN : CLONING AND SEQUENCING OF 3ABC GENE OF FOOT AND MOUTH DISEASE VIRUS TYPE O FROM A THAI PIG ISOLATE FROM RATCHABURI PROVINCE IN 2005. ADVISOR : ASSOC. PROF. ATHIPOO NUNTAPRASERT, D.V.M., Ph.D., 61 pp.

Foot and mouth disease (FMD) is an important epidemic disease of swine in Thailand. It causes negative effect on swine production and also creates export trade restriction. Consequently, the economic loss is enormous in swine industries. The major type of Foot and mouth disease virus (FMDV) found in Thailand and has been recently isolated from an outbreak in 2005 is FMDV type O. To control this disease is to distinguish the naturally infected swine from the vaccinated ones. In this study, viral RNA was extracted from vesicular fluid and erosion tissue found in coronary band of hoof in FMD infected pigs from Ratchaburi province in 2005. The first strand of FMDV-cDNA was synthesized by reverse transcription reaction. FMD Type O was confirmed by PCR technique using the O-1C_{124} primer set. Then, the full length of 3ABC gene was amplified by using PCR technique. The PCR products of 3ABC gene were cloned into pET160/GW/TOPO® vector. In addition, the nucleotide sequences were analyzed and compared with previously reported 3ABC nucleotide's databases. The results showed that five clones of 3ABC expression vectors were successfully collected (1,303 bp). As a result, phylogenetic tree analysis of 3ABC nucleotides revealed that this gene of FMDV type O isolated from pig in Ratchaburi province in 2005 had 100 % homology to those of FMDV type O strain Tibet /CHA/99 (Genbank accession number is AJ539138).

Department : Veterinary Medicine	Student's Signature K. Marupanthorn
Field of Study : Veterinary Medicine	Advisor's Signature
Academic Year : 2008	Co-Advisor's Signature

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	base pair
ºC	degree Celsius
cm	centimeter
cDNA	complementary deoxyribonucleic acid
DNA	deoxynucleic acid
dNTPs	dATP, dTTP, dGTP, dCTP
dpi	day post infection
DW	distilled water
e.g.	exempli gratia, for example
EDTA	ethylenediamine tetraacetic acid
E.coli	*Escherichia coli*
et al.	Et alii, and others
g	gram (s)
h	hour (s)
i.e.	id est, that is
kb	kilobase
LB	Luria-Bertani media
M	molar
mg	milligram (s)
$MgCl_2$	magnesium chloride
min	minute (s)
µl	microliter
µM	micromolar
ml	milliliter
mM	millimolar
ng	nanogram
nm	nanometer
OD	optical density
PCR	Polymerase Chain Reaction
pH	the negative logarithm of hydrogen ion concentration
RNase	ribonuclease
rpm	revolution per minute
sec	second (s)

TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
U	unit
UV	ultraviolet

CHAPTER I

INTRODUCTION

Foot and mouth disease (FMD) is an acute, highly contagious and economically devastating viral disease of cloven-hoofed animals. It plays an important role in livestock and causes economic loss due to lower production and international trade restriction. Currently, this disease is present in two-thirds of the OIE (Office International des Epizooties) member countries where it creates severe economic problems and provides a reservoir of virus ready to spread into virus free areas. A recent study on the economic impact of a FMD outbreak in the United States concluded that larger impact of this disease caused the loss of export markets and consumer fears (Paarlberg et al., 2002). Similar conclusions have been reported from an outbreak of FMD in Thailand (Linchongsubongkoch, 2003).

Foot and mouth disease virus (FMDV) is divided into 7 distinct serotypes (A, Asia1, C, O, SAT1, SAT2 and SAT3) based on complete loop of cross protection. Type O, A and Asia1 are considered as endemic serotypes in domestic animals in Thailand (Office International des Epizooties, 2005) and the most of FMDV infection in pig is FMDV type O (Knowles et al., 2005). In Thailand, a nationwide FMD epidemic is ongoing under control strategies. The number of FMDV type O infected pig was 261 pigs and dead pigs was 111 pigs in 2004 (Department of Livestock Development: DLD, 2004[a]; 2004[b]). In 2005, the number of FMDV type O infected pig was 349 pigs and dead pigs was 12 pigs in 2005 (DLD, 2005[a]; 2005[b]; 2005[c]). There was no officially reported in 2006 (DLD, 2006). However, the number of FMDV infected carrier pig has never been reported in Thailand and a little known about the study of FMD typing from field isolated samples.

FMDV is a single-stranded RNA virus belonging to the genus *Aphtovirus* in the family *Picornaviridae*. This genus also includes equine rhinitis A virus (ERAV), previously known as equine rhinovirus type 1 (Hartley et al., 2001). Like other picornaviruses, FMDV genome consists of single-stranded linear RNA with an approximate size of 8 kb (Forss et al., 1984; Kweon et al., 2002). The RNA of FMDV has a single open reading frame (ORF) encoding one long polypeptide that is processed to 12 viral proteins. Among the viral proteins induced by FMDV infection, four proteins (VP1–4) are the major subunit of viral capsid. Although the other proteins like L, 2A–C and 3A–D are not part of capsid structure, they also

induce antibody responses in infected animals (Berger et al., 1990; Diego et al., 1997; Lubroth and Brown., 1995; Malirat et al., 1998; Rodriguez et al., 1994; Silberstein et al., 1997)

There are three particular features of FMD which make rapid, sensitive, and specific laboratory tests essential for diagnosis. Firstly, it is the most contagious disease of cloven-hoofed animals; hence it has the potential for explosive spread in susceptible livestock populations. Secondly, it is acknowledged as the most serious constraint to international trade in livestock and animal products. Thirdly, other viruses can produce symptoms which are clinically indistinguishable from FMD. Therefore, necessary laboratory investigation for a definitive diagnosis is required (Reid et al., 2001). Traditionally, primary virus diagnosis of FMD is carried out by complement fixation test (CFT) or by enzyme linked immunosorbent assay (ELISA) on epithelial tissue suspension or vesicular fluid of clinical samples. These suspensions are simultaneously inoculated into cell culture and the specificity and serotype of isolated viruses is further established by the CFT or the ELISA, sandwich ELISA for FMDV typing and liquid phase block ELISA for screening of FMDV antibody titer. However, these diagnostic methods cause delay many times, have high cost, and need intensive labor. Recent advances in molecular biology have resulted in the development of techniques such as reverse-transcriptase polymerase chain reaction (RT-PCR) for detection of FMD genomic RNA in culture fluid or in clinical samples (Remond et al., 2002). Although various protocols have been tested, none of them is separately used for primary diagnosis but for conjunction with the standard methods.

FMD Vaccine is used for control outbreaks of disease and is a dead preparation of whole virus particles in an oil or aluminum hydroxide/saponin adjuvant. There will be no replication of the virus following vaccination, and the vaccinated animal will develop antibodies to structure of viral capsid proteins. Some of virus can neutralize, and protect it from subsequent infection. There is no viral replication and no expression of the non structural proteins (NSPs). The animal will not develop antibodies particularly against 3D to those proteins. Although some vaccines contain low levels of these NSPs depending on the manufacturing process, those vaccines stimulate low antibody response to the NSPs especially more obvious in multiply vaccinated animals. The 3D gene encodes for the viral polymerase, and antibody response to this protein were detected in the agar gel immuno-diffusion VIAA (virus infection associated antigen) test (Kitching, 2004).

Recombinant antigens are brought into attention because of their specificity and reproducibility of quality of prepared antigens. Recombinant antigen based on serologic tests are widely used in screening for FMDV infection in cattle, but use of recombinant proteins for serodiagnosis of FMDV in pig has not been widely investigated. Many diagnostic tests have been developed to distinguish infected animals from vaccinated animals using detection of antibodies to the NSPs especially in 3ABC gene (Kweon et al., 2003; Official of Agricultural commodity and food standard, 2004). It is also known that currently available inactivated vaccines mainly consist of capsid proteins and induce weak antibody responses to NSPs in vivo after vaccination, whereas infected animals produce antibodies to both structural and NSPs proteins (Diego et al., 1997; Mackay et al., 1998). Assays demonstrating antibodies against NSPs have potential to differentiate infected animals from those of merely vaccinated animals (Sorensen et al., 1998; Clavijo et al., 2004[b]).

Furthermore, Clavijo et al. (2004[b]) reported that antibody response to 3ABC protein in cattle, sheep and pigs was detected as early as 7 dpi. Eble et al. (2007) reported antibody response to 3ABC protein was detected in 8 dpi in vaccinated pig. The profiling ELISA showed that antibody response to 3ABC appeared early after infection and antibody to 3ABC could be detected for longer than antibody to any other NSPs (Mackay et al., 1998). Thus, the several ELISAs have been developed to distinguish infected animals from those of vaccinated animals by using 3ABC protein.

The advent of biotechnology has made possible immunoassay to 3ABC recombinant protein by enabling their production in large quantities through in vitro expression in a variety of prokaryotic or eukaryotic vectors. A wide range of 3ABC gene had been expressed as fusion proteins in *E. coli* or using baculovirus vector in insect cells (Remond et al., 2002). This research cloned 3ABC gene into *E. coli* which had a simple cultivation and large amounts of recombinant protein but lacked eukaryotic post-translational modifications (Turner et al., 2005). Fortunately, the post-translational modification is not nessary for the 3ABC gene (La Torre et al., 1980).

Therefore, the purposes of this research are to clone 3ABC gene of FMDV type O isolated from a Thai pig in Ratchaburi province in 2005 into a bacterial expression vector, to study the sequence of 3ABC gene and the possibility of gene variation. The 3ABC gene was amplified from FMDV cDNA by using polymerase chain reaction (PCR) with primers specific for 3ABC gene of FMDV type O. The plasmid pET160/GW/D-TOPO containing sequence of histidine residues was used for constructing a plasmid expressing 3ABC protein.

PCR product and pET160/GW/D-TOPO plasmid were cut with appropriate enzymes and the digested products were ligated. The expression plasmid was transformed into *E.coli.* Single clone was selected and confirmed by using PCR and DNA sequencing. In addition, the obtained sequence was compared and analyzed the homology data with the previously reported 3ABC sequence database from Genbank as follow; O1Campos (Genbank accession number is AJ320488), O1manisa iso87 (Genbank accession number is AY593823), Taiwan97 iso106/112 (Genbank accession number is AY593835), Tibet/CHA/99 (Genbank accession is number AJ539138), and UK2001 iso84 (Genbank accession number is AY593836) using Bioedit Sequence Alignment Editor (Hall, 1999) and MEGA version 4.1 (Tamura et al., 2007) softwares.

The sequencing data of 3ABC gene is the first report in Thailand. It is compared with other 3ABC gene of FMDV type O database from Genbank. In addition, both data and techniques used in this study can be applied to further studies involving cloning of 3ABC FMDV gene in the other type of FMDV and other virus.

CHAPTER II

REVIEW LITERATURES

1. General characteristics and pathogenesis of FMDV

FMDV is a single-stranded RNA virus belongs to the genus *Aphtovirus* in the family Picornaviridae. The only other member of this genus is equine rhinitis A virus (ERAV) (Grubman and Baxt, 2004). 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).

Disease is characterized by fever and vesicular erosion on mouth, tongue, muzzle, hooves and udder which leads to salivation and lameness (Figure 2.1). The morbility rate is very high but the mortality rate is low except in very young animals (Linchongsubongkoch, 2003). In young piglets, the infection may be fatal due to myocarditis. Pigs usually become infected by eat FMDV-contaminated feed, direct contact with infected animals, or by being placed into areas that had once housed FMDV-infected animals. However, pigs are much less susceptible to aerosol infection than cattle (Alexandersen et al., 2002), yet they excrete far more aerosolized virus than cattle or sheep. In cattle, the incubation period is depends on the amount of infecting virus and route of infection, but it is generally 2 days or more. The infected animals develop fever, viremia, and lesions on the feet and tongue. Foot lesions are the most common finding in pigs, while lesions at other sites occur less frequently. Tongue lesions are usually small and less noticeable than those of in cattle (Kitching and Alexandersen, 2002). In young piglets, the infection may be fatal due to myocarditis. Initial replication of the virus occurs at the site through which virus gains entry, followed by rapid dissemination to most of the epithelial sites within the animal (Brown et al., 1995). Interestingly, virus can be found at sites where clinical lesions either are not present or do not form (Oleksiewicz et al., 2001). While pigs excrete large amounts of aerosolized virus, recent evidence suggests that much more viral replication takes place in the nasal mucosa than in the lungs (Grubman and Baxt, 2004).

A. B.

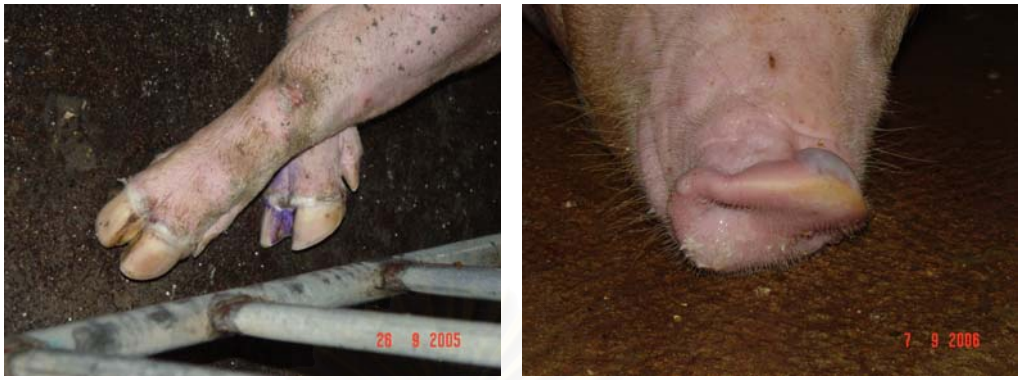


Figure 2.1 Generalized lesions in pigs affected with FMDV, field cases. A. lesions are evident along the coronary bands and B. lesions at the snout. Courtesy of Dr. Supol Luengyosluechakul.

The virus replicates to a high titer in epithelial cells, particularly those undergoing repair, and consequently lesions may also be seen on the hocks or elbows of pigs being housed on concrete floor where damage to legs are common. FMDV also destroy the replicating myocardial cells of young susceptible species, resulting in high mortality from heart failure.

Susceptible livestock may be infected by FMDV as a result of direct or indirect contact with infected animals or with an infected environment. When infected and susceptible animals are in close proximity, the aerial transfer of droplets and droplet nuclei is probably the most common mode of transmission. Long-range airborne transmission of virus is an uncommon but important route of infection, requiring the chance combination of particular factors, including (1) the animal species, (2) the number and location of the transmitting and recipient animals, and (3) favourable topographical and meteorological conditions (Alexanderson et al., 2003).

Eventhough FMD infected animals are slaughtered, FMDV still alive in these frozen carcasses until rigor mortis. If some infected products are fed to susceptible species (e.g. pigs), an outbreak of FMD will be likely to occur. There are remain numerous examples of FMD outbreaks initiated through ingestion of FMD virus-infected products by pigs (Kitching, 1998); hence countries frequently maintain strict regulations concerning the heat treatment of pig swill.

FMDV is particularly susceptible to inactivation outside its host, for example exposure to high temperatures, drying or where the pH is <6 or >10. When a carcass is permitted to mature after slaughtering (at 2 °C for 24 h), the lactic acid will kill any virus in the meat by reducing the pH to <6. No reduction in pH occurs in the glands or bone marrow; however, with certain safeguards, it is possible to safely import meat off the bone from countries where FMD is present. Milk from infected animals will also contain large quantities of live virus, sufficient to infect calves or pigs (if the milk is inadequately heat-treated or not diluted by uninfected milk). Semen from infected bulls and ova from infected cows may also be contaminated with live virus (Kitching et al., 2005).

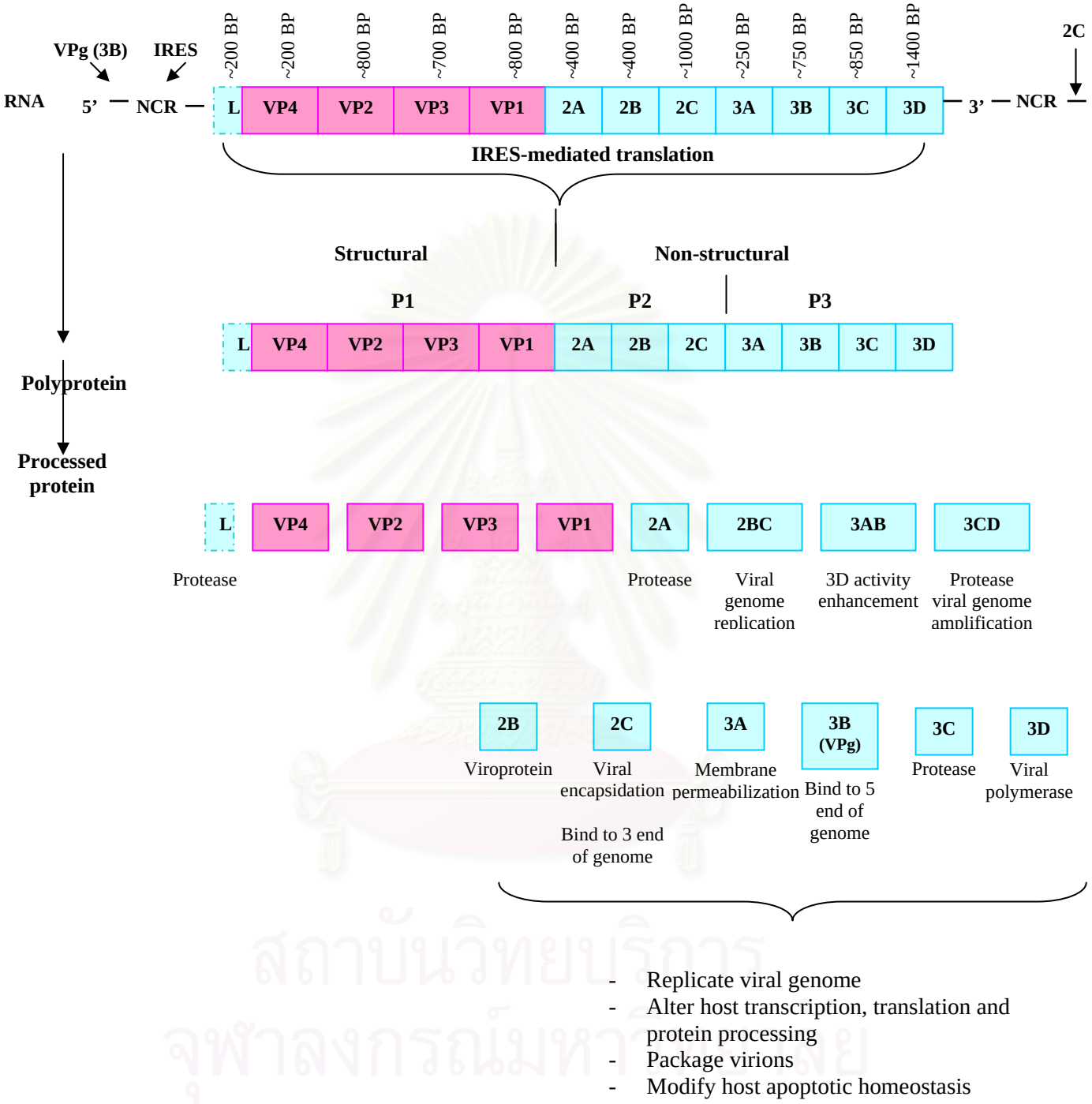
2. Genome organization

The virion is a 140S particle consisting of a single-stranded RNA genome and 60 copies each of four structural proteins (VP1 [1D], VP2 [1B], VP3 [1C], and VP4 [1A]). The FMDV genome has a basic organization similar to those of other members of the *Picornaviridae*, and the nomenclature for the viral proteins was established by Rueckert and Wimmer (1984). Within the virion, there are small amounts of a cleavage precursor of VP2 and VP4, called VP0 (1AB), and one copy of a 23 to 24-amino-acid genome-linked protein, 3B (VPg), covalently bound to the 5' terminus of the RNA (Grubman, 1980). The organization of the viral genome is shown in Figure 2. The RNA is translated as a single long open reading frame (ORF) into a polyprotein, followed by a series of posttranslational proteolytic cleavages to generate both the intermediate and mature structural and NS viral proteins (Grubman et al., 1984; Robertson et al., 1985).

Based on the initial cleavage products, the genome ORF is divided into four regions (Figure 2.2). The 5' end, the L region, which encodes the N-terminal component of the polyprotein, contains two in-frame AUG initiation codons that result in the generation of two L proteins, Lab and Lb (Sangar et al., 1987). While both forms of L are synthesized during in vitro translation of viral RNA (Beck et al., 1983) and in infected cells (Clarke et al., 1985), it has been shown, by using site-directed mutagenesis, that deletion of the second AUG from an FMDV infectious clone abolished viral replication upon transfection of the transcribed RNA into cells, while deletion of the first AUG had no effect on viral replication (Cao et al., 1995). The L protein, a papain-like protease (L^{pro}) (Piccone et al., 1995; Robertsand Belsham, 1995), is autocatalytically cleaved from the polyprotein at its C terminus (Strebel and Beck, 1986).

The L^{pro} also plays a role in inhibition of host protein synthesis and has been identified as a viral virulence factor.

Directly downstream of the L region is the P1 region of the genome (Figure 2.2), encoding the four viral structural proteins VP4, VP2, VP3, and VP1. Following the P1 region is the P2 region (Figure 2.2), encoding three viral NS proteins, 2A, 2B, and 2C, and the P3 region, encoding NSPs 3A, three copies of VPg, $3C^{pro}$, and $3D^{pol}$. Historically the 2A region was considered part of the P2 region; however, genetic and biochemical evidence has shown that the FMDV 2A peptide is cleaved as a P1-2A precursor (Vakharia et al., 1987). $3C^{pro}$ is identified as a viral protease (Klump et al., 1984) and is involved in processing the viral polyprotein, while 3Dpol is the viral RNA dependent RNA polymerase (Cowan and Graves, 1966; Lowe and Brown, 1981; Newman et al., 1979).



- Replicate viral genome
- Alter host transcription, translation and protein processing
- Package virions
- Modify host apoptotic homeostasis

Figure 2.2 Schematic map of the FMDV genome. Also shown are the functional elements of the genome as described in the text and the partial protein cleavage products.

3. Function and application of FMDV genes

The FMDV genome is composed of three parts, the 5′ untranslated region (5′ UTR), the coding region and the 3′ untranslated region (3′ UTR) containing a heteropolymeric segment and a poly (A) tail, which is required for viral replication. FMDV also contains an internal poly(C) tract within the 5′ UTR. After translation, the polyprotein is cleaved into four primary cleavage products: (1) the amino terminal L protease which cleaves at its own carboxyl terminus; (2) P1 (VP1, VP2, VP3, and VP4) - 2A, the precursor of the capsid proteins; (3) 2BC, and (4) P3, which is cleaved to make the replicative or NSPs 3A, 3B, 3C and 3D, the RNA-dependent-RNA polymerase (Belsham, 1993).

The icosahedral symmetrical viral capsid is assembled from the four structural proteins, with VP1 being the most important because of its dual function in cell receptor binding and antigenic determination (Mateu, 1995; Feng et al., 2004) Currently, much research effort has been focusing on the sequence analysis of structural proteins, especially VP1, owing to their dominant roles in antigenic/serotype determination (Kitson et al., 1990). In the intervening years the great deal has been learned about the function of VP1, the principle target sequence for the development of synthetic vaccines, both in its role as the receptor binding ligand of the virus and as an antigenic determinant (Rowlands, 2004).

The VP2, VP3 and VP4 gene are the part of viral capsid, which VP4 is a major protein to stable a viral capsid. L and 2A gene are the major proteinases for hydrolytic cleavage of the polyprotein (Klump et al., 1984; Strebel and Beck, 1986). VP2, VP3, VP4, L and 2A protein are not used in any application. The function of 2B gene is amplifying RNA, inhibit alteration of membrane permeability of cellular exocytosis and rearrange endoplasmid reticulum. The short hairpin RNA targeted to the highly conserved 2B NSP coding region is developed to inhibit replication of multiple serotypes of FMDV (Santos et al., 2005). The function of 2C gene is formation of vesicles, virus encepsidation and RNA binding in RNA replication. The 3A gene has been found to be associated with host alteration (Beard and Mason, 2000; Knowles et al., 2001) and inhibit MHC class I expression (Clavijo et al., 2004[a]). The 3B (VPg) gene, also known as the genome-linked protein, may be involved in priming RNA replication (Wimmer, 1982). The 3C gene is responsible for proteolytic cleavage of the majority of the cleavage sites in the FMDV polyprotein. The 3D gene, the viral RNA-dependent RNA polymerase (RdRp), is responsible for both positive- and negative-sense RNA replication. The recently resolved crystal structure of the poliovirus 3D and other genetic/biochemical analyses suggest that the polymerase might function as a

higher order oligomeric structure (Hobson et al., 2001). The 2C, 3ABC and 3D gene are applied to develop recombinant protein for ELISA test kit to distinguish FMDV vaccinated animals from FMDV infected animals.

In addition, the viral proteinases L, 3C and the 2A oligopeptide discussed previously are the NSPs from the subregion 2BC, with various not well defined functions. The 3A, 3B and 3D genes are primarily involved in polyprotein processing and viral RNA replication, as shown in 2.1.

Table 2.1 Function and application of FMDV genes (Clavijo et al. 2004[a])

Genes	Function	Application
VP1	location of viral antigenic determinant	synthetic peptide FMD vaccine typing of FMDV
VP2	capsid protein	-
VP3	capsid protein	-
VP4	major stability of capsid protein	-
L	host protein synthesis shut off protease cleavage	-
2A	protein cleavage (polypeptide)	-
2B	alteration of membrane permeability inhibition dissociation/rearrangement of endoplasmid reticulum and Golgi RNA amplification	short hairpin RNA inhibit replication of FMDV
2C	formation of vesicle virus encapsidation RNA binding in RNA replication (as 2BC)	recombinant protein antigen for ELISA
3A	inhibit MHC class I expression inhibition of cellular protein secretion virus interaction with host cells and host range	recombinant protein antigen for ELISA
3B	primer of RNA synthesis membrane association of replication complexes (as 3AB)	recombinant protein antigen for ELISA
3C	viral protein processing host protein cleavage	recombinant protein antigen for ELISA
3D	RNA dependent RNA polymerase stimulation of RNA synthesis	recombinant protein antigen for ELISA

Upon infection, FMDV elicits a rapid and broad spectrum humoral and cellular response that will protect against infection with homologous and antigenically related viruses. However, immunity to FMDV is thought to be primarily humoral. Infection of susceptible cattle with FMDV results in a rapid rise of serum-neutralizing antibody which can be detected from 4–5 days post-infection and peak at around 28 days. The development of this serum response coincides with resolution of lesions, termination of viremia and the reduction of virus excretion (Doel, 1996). For successful clearance of virus, phagocytosis is assisted by antibody-dependent opsonisation of virus (Rigden et al., 2002). There is evidence of the importance of T helper lymphocytes in the development of the immune response to FMDV infection. However, early events after FMDV infection could prevent the expression of MHC class I peptide complexes on the plasma membrane, which is enhanced by the subsequent virus-induced shut-off of cellular protein synthesis, resulting in a rapid reduction of class I expression in the surface of FMDV infected cells. This inhibitory mechanism affects the presentation of viral peptides by FMDV infected cells to cytotoxic T lymphocytes (Sanz-Parra et al., 1998). The immune response to FMDV is affected by host and viral variables. Host variables include species, breed, age, health, physiological states and FMD immune status. Viral factors including dose, route, volume and viral strain amongst others affect the host response to FMDV infection (Doel, 1996). Both structural and non-structural antigens induce the production of antibodies in infected animals. Infected animals are exposed to NSPs at the time of cytolysis, which because of the replication strategy of the virus are produced in equimolar amounts in infected cattle.

In contrast, vaccinated animals which have not been exposed to replicating virus will develop antibodies only to the viral antigens in the inactivated material. Vaccines consist of semi-purified, chemically inactivated virus and elicit antibodies principally to structural proteins. However, some FMDV vaccines contains NSPs in addition to inactivated virions, and depending on their concentration, they may induce a detectable immune response. The virus-infection associated antigen, VIAA, isolated as a crude antigen extract from tissue culture reacts in agar gel with sera from convalescent animals (Cowan and Graves, 1966). However, VIAA or NSP 3D is present both in the tissue culture from which vaccines are prepared (Donnell et al., 1997) and in the viral particle. Therefore, VIAA antibodies are also present in the sera of vaccinated animals. However, in general no VIAA antibodies are detectable following the initial vaccination, but are not unusual in the sera of animals which have been given multiple vaccinations. This immune response usually disappears within 60–90 days post-revaccination as detected by AGID or 90–180 days post-revaccination when ELISA-3D is used (Donnell et al., 1997). As a consequence, other groups have made efforts

to identify antibodies to the NSPs which could provide a more reliable indicator of infection. In a series of experiments, Berger et al. (1990) analyzed bovine hyperimmune, convalescent sera for FMD-specific antibodies. The sera clearly differed from post-vaccination sera by their capacity to precipitate NSPs 3AB and/or 3C, 2C, and occasionally 3A and 2B. It was concluded that animals which had recovered from FMD would be identified by the presence of antibodies to NSPs. Simultaneous detection of at least two NSP antibodies (excluding the NSP 3D) would be sufficient to demonstrate viral replication (Berger et al., 1990). Further experiments found that the presence of antibodies to the 2C protein, and to a lesser extent to the polypeptide 3ABC, could be used to differentiate the potential carrier convalescent animal from the vaccinated animal and antibodies response to the 2C protein could be detected in cattle up to 365 days after infection (Lubroth and Brown, 1995). The absence of 2C antibodies from the sera of vaccinated animals was explained by the association of this viral protein with cellular debris which is separated from the virus harvest prior to inactivation of the supernatant for vaccine production (Lubroth et al., 1996).

4. The study of NSPs

The diagnostic challenge is to distinguish infected animals, either vaccinated or unvaccinated, from those that have been only vaccinated against the disease since both groups have neutralizing antibodies in their sera. Several ELISAs have been developed to distinguish infected animals from those that have been vaccinated, all based on the detection of antibodies to the NSPs of FMD virus (Table 2.2). Tests to detect antibodies to the polyprotein 3ABC have so far been the most successful. This virus specific NSP has been produced either in recombinant *E. coli* (Strebel and Beck, 1986) or in insect cells infected by appropriately recombinant baculovirus (Sorensen et al., 1998).

Table 2.2 Summary of recombinant NSPs-base diagnostic test

Antigen(s)	Dignostic test	System	Plasmid vector	Remark	Reference
2C	I-ELISA	Baculovirus	pVL1392 (Pharmingen,USA)	suitable antigen for the development of a diagnostic test	Meyer et al., 1997
3AB	LPB-ELISA	Baculovirus	pVL1393 (Pharmingen,USA)	-	Sorensen et al., 1998
3AB	I-ELISA	Baculovirus	pBAcPAK8 (Invitrogen, USA)	lower silkworm larvae protein in lysates of insect cells	López et al., 2005
3ABC	I-ELISA	*E. coli*	pBacPAK9 (Clontech, USA)	-	Kweon et al., 2003
3ABC	SPB-ELISA	*E. coli*	pET30c(+) (Novagen, USA)	-	Clavijo et al., 2004[b]
3ABC	LPB-ELISA	*E. coli*	pMD18-T (Takara, Japan)	-	Sun et al., 2004
3ABC	LPB-ELISA	*E. coli*	pTriEx-4Neo (Novagen, USA)	-	Lu et al., 2007

Table 2.2 Summary of recombinant NSPs-base diagnostic test (Continue)

Antigen(s)	Test	System	Plasmid vector	Remark	Reference
3ABC	LPB-ELISA	Baculovirus	pVL1393 (Pharmingen,USA)	react with sera from vaccinated animals	Sorensen et al., 1998
3D	I-ELISA	*E. coli*	pGEX-3X (Pharmacia)	react with sera from vaccinated animals	Newman et al., 1994
3D	I-ELISA	Baculovirus	pVL1393 (Pharmingen,USA)	react with sera from vaccinated animals	Meyer et al., 1997
3D	LPB-ELISA	Baculovirus	pVL1393 (Pharmingen,USA)	react with sera from vaccinated animals	Sorensen et al., 1998

Note: LPB-ELISA, liquid phase blocking ELISA; SLB-ELISA, solid phase blocking ELISA; I-ELISA, indirect ELISA.

In order to reduce the number of false positives due to the presence in the sera of antibodies against expression vector antigens (particularly *E. coli*-derived recombinant proteins), a confirmatory test can be included in the testing format or recombinant proteins can be captured with a specific monoclonal antibody. An Enzyme-linked immunoelectrotransfer blot assay (EITB) has been used as a confirmatory test in combination with a 3ABC ELISA in order to reduce the number of false positives (Bergmann et al., 2000). This approach has been used as part of the FMD eradication program in South America during the past decade. The procedure adopted in sero-surveillance for evidence of infection in Brazil, for example, has been to screen sera from vaccinated cattle using the 3ABC test and retest positive samples by the EITB. If still positive, the animal from which the serum was collected is re-sampled three months later, prior to its next vaccination, and only if this sample is positive by the EITB, are further investigations initiated (Diego et al., 1997) and developed an indirect-trapping ELISA based on the capture of semi-purified 3ABC recombinant protein by a monoclonal antibody.

Another alternative has been the use of peptides for the identification of anti-NSP antibodies identified continuous antigenic determinants within the amino acid sequences of the NSPs 2C and 3ABC. Based on these studies peptides for the 3A and 3B NSP were considered candidates for the specific differentiation of vaccinated from convalescent animals. The 3B protein was chosen because of its greater specificity when compared with the 3A protein that reacted with some sera of vaccinated animals (Shen et al., 1999).

In general, NSP-tests have been predominantly designed to detect NSP antibodies in cattle and are less useful in sheep and pigs. Sheep, in particular, probably because of the frequently subclinical nature of the disease, may fail to develop detectable levels of these antibodies. Therefore, the severity of the infection is likely to be the major influence on the levels and the subsequent duration of detection of NSP antibodies.

An additional problem, not fully addressed by existing tests for NSP antibodies, is the detection of vaccinated animals which have contact with live FMD virus and become virus carriers. Because of the protection provided by the vaccine, there may only be limited

replication of the virus and very low levels of NSP expression and therefore antibody production by the infected animal, thus exceeding the sensitivity of the tests currently available. An NSP ELISA was not capable of detecting antibodies in all tested carrier animals when sera was examined for antibodies to NSPs L, 2C, 3A, 3D and 3ABC by an indirect ELISA profiling (Mackay et al., 1998).

A considerable variation was observed in the antibody response to NSPs of both naïve and vaccinated animals following infection. Animals that showed clinical signs but eliminated the virus are as likely to be sero-positive several months after infection as clinically affected animals which remain persistently infected. Therefore, the NSP tests should not be used on an individual basis but on a herd basis with an appropriate sampling schedule, which includes the younger population of animals born at the time when transmission was not expected to occur. It can confirm the absence of viral activity in the herd. NSP tests have been shown to be useful in detecting viral activity in naïve or vaccinated populations, but may not be a useful indicator of the current virological status of an individual animal. In herds vaccinated with potent FMD vaccines, some animals that are exposed to live virus will support replicating virus as vaccines do not induce full herd protection. Although these animals may not present any clinical signs, they will seroconvert to some NSPs. Therefore, testing the entire herd should detect previous viral activity and determine the potential for the presence of carrier animals (Clavijo et al., 2004[a]).

CHAPTER III

MATERIALS AND METHODS

In this study, the author divided the study into 2 phases. In phase I, the confirmation of FMDV type O was performed using RT-PCR, and in phase II, the 3ABC gene of FMDV type O was cloned into a bacterial expression vector and analyzed by DNA sequencing. The conceptual framework is shown in Figure 3.1

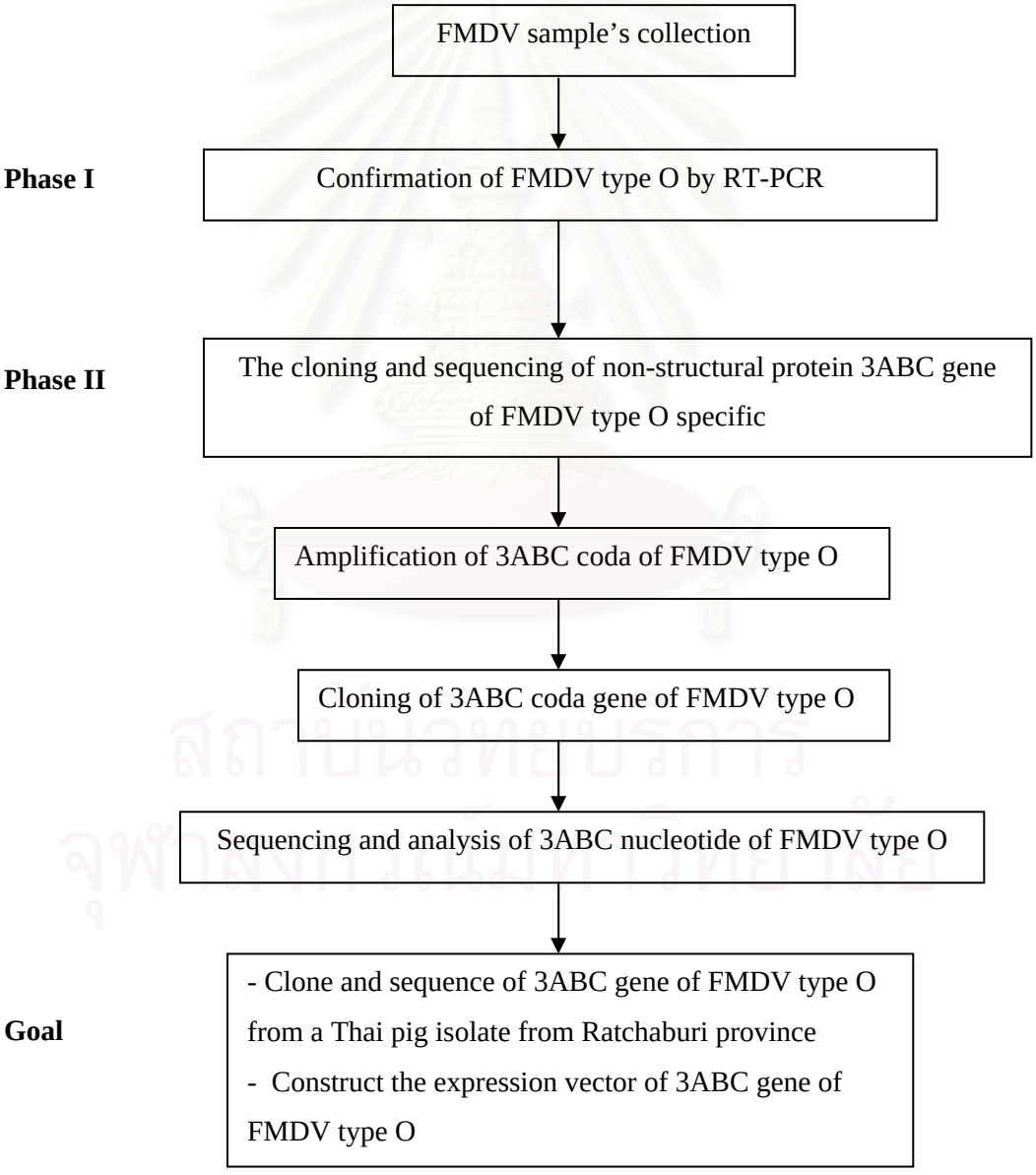


Figure 3.1 The conceptual framework in this study

FMDV sample's collection

A total of five FMDV outbreak's farms were selected in this study. The vesicular fluid and erosion tissue samples of FMDV infected pigs were collected in 10% glycerine buffer from pig farms. All FMDV samples were stored at -80 °C until further study.Those farms which reported the incidence of FMDV type O outbreak in 2005 were located in Nakhon pathom (1 sample), Phetchaburi (2 samples), and Ratchaburi province (2 samples) (Department of Livestock Development. 2005[b]). The FMDV stock from Ratchaburi province was selected to clone into a bacterial expression vector because the Ratchaburi province is the first province that officially reported incidence of FMD outbreak in 2005.

Phase I Confirmation of FMDV type O by RT-PCR

All of the farm pig samples were extracted for total viral RNA. The FMDV O-1C124 and 3ABC cDNA were synthesized. The PCR with type O specific primers were analyzed.

1. FMD viral RNA extraction

Total RNA of samples was isolated from farm pig samples and from killed vaccine (3 types; type O, A, and Asia1, DLD, Thailand) using Purelink® viral RNA/DNA mini kit under construction from manufacturer (Invitrogen, USA). The samples were added with 25 µl of Proteinase K and 200 µl of Lysis Buffer (containing 5.6 µg Carrier RNA), and slightly homogenized. The homogenate was incubated at 56 °C for 15 minutes. Consequentially, 250 µl of 95% ethanol was added and vigorously vortexing for 15 seconds. The mixture was incubated at room temperature for 5 minutes and centrifuged briefly to remove any drops from the inside of the lid. The lysate was transferred to the Viral Spin Column (Invitrogen, USA). Then, the pellet was collected by centrifugation at about 6,800 x g for a minute. The total RNA was dissolved with 20 µl of Sterile, RNase-free water and kelp at -80°C.

2. First-stranded cDNA synthesis

The first-stranded cDNA was generated using Omniscript Reverse Transcription kit under construction from manufacturer (Qiagen, Germany). Two micrograms of the total RNA sample was added into the mixture (20 µl of final volume) of 0.1 µM of oligo (dT) primers, 1x of buffer RT, 2 µl of the dNTP mix (5 mM of each dATP, dCTP, dGTP, and dTTP), 1µl of

reverse transcriptase (200 units/µl), 0.5 µl of recombinant RNasin® ribonuclease inhibitor (20U), and RNase-free water; respectively. The reaction was incubated at 37 °C for 1 hour and immediately incubated at 93 ºC for 5 minutes to terminate the reverse transcription activity. The cDNA was stored at -20 ºC until used.

3. Confirmation of FMDV type O (O-1C$_{124}$) by RT-PCR

The samples from infected farm pigs were separated into two parts. The first part was sent to confirm typing at Regional Reference Laboratory for Foot and Mouth disease in South East Asia (RRL), Nakhon Ratchasima, Thailand. The second part was confirmed of FMDV typing by using RT-PCR at Department of Veterinary Medicine, Chulalongkorn University, Bangkok Thailand.

The PCR reactions were contained 50 µl of the mixture as follwed: 5 µl of cDNA, 2x PCR buffer, 400 µM dNTPs, 7.5 mM $MgCl_2$, 20 pmole of each primer, and 1 U *pfu* DNA polymerase. PCR amplifications were conducted on a PCR T-gradient Thermoblock® (Biometra®, Germany). PCR thermocycing conditions were an initial denaturation at 94°C for 4 minutes, and 40 cycles of denaturation for 1 minute at 94°C, primer annealing for 1 minute at 50°C, and DNA extension for 90 seconds at 72°C and a final extention at 72°C for 5 minutes. PCR products were visualized on 2% agarose. The PCR products size of FMDV type O were approximately at the molecular weight of 1301 bp. Gel were purified using QIAQuick Gel Extraction kit (Qiagen, Germany). Representative DNA samples were submitted for sequencing at 1st base Inc. (Singapore). The primers used for confirmation of FMDV typing is listed in Table 3.1

Table 3.1 The O-1C_{124} primers used for confirmation of FMDV typing (Knowles and Samual, 1994)

Primer designation	Sequence (5'-3')	Amplicon size (bp)
O-1C_{124}-F	ACCAACCTCCTTGATGTGGCT	1301
O-1C_{124}-R (NK61)	GACATGTCCTCCTGCATCTG	

Phase II The cloning and sequencing of non-structural protein 3ABC gene of FMDV type O specific

The experiment in this phase included 1) amplification of 3ABC gene of FMDV type O by RT-PCR, 2) cloning of 3ABC cDNA and 3) sequencing and analysis of 3ABC nucleotide.

1. Amplification of 3ABC gene of FMDV by RT-PCR

The PCR product from the FMDV was used as template for PCR amplification of the 3ABC gene with a pair of degenerate oligonucleotide primers. The two primers were designed based on the 3ABC gene from FMDV type O strain Tibet/CHA/99 (Genbank accession number is AJ539138), which has the same similarity to the previous report from Genbank of 3ABC FMDV type O in Thailand. The cloning sites were incorporated into the forward and reverse primers. Their sequences were as follows: forward primer, 3ABC-F: 5' CACC CAA TTC CTT CCC AAA AGG CT 3' and reverse primer, 3ABC-R: 5' GTG GTG TGG TTC GGG GTC AA 3'. Locations on the corresponding genes of 2 primers designed in this study are shown in Appendix C.

The PCR reactions were contained 50 µl of the mixture as follwed: 2x PCR buffer, 400 µM dNTPs, 7.5 mM $MgCl_2$, 20 pmole of each primer, and 1 U *pfu* DNA polymerase. PCR amplifications were conducted on a PCR T-gradient Thermoblock® (Biometra®, Germany). PCR thermocycing conditions were an initial denaturation at 94°C for 4 minutes, and 40 cycles of denaturation for 1 minute at 94°C, primer annealing for 1 minute at 50°C, and DNA extension for 90 seconds at 72°C and a final extension at 72°C for 5 minutes. PCR

products were visualized on 2% agarose. The size of 3ABC gene of FMDV type O PCR product was approximately 1303 bp. Gel were purified using QIAQuick Gel Extraction kit (Qiagen, Germany).

2. Cloning of 3ABC cDNA into pET160/GW/D-TOPO vector

After gel extraction, the 3ABC gene was cloned into the pET160/GW/D-TOPO (His tag) vector (Invitrogen, USA), named as 3ABC recombinant plasmid. The 3ABC recombinant plasmid was transformed into an *E.coli* strain One Shot® TOP10 (Invitrogen, USA) using chemical transformation. The transformants were selected on the ampicillin LB agar plates. Then, the positive transformants were analyzed by using PCR. For PCR primers, use a combination of the T7 Promoter sequencing primer and 3ABC-R primer. The sizes of transformant PCR product was approximately 1524 bp in length, respectively. A single colony of *E. coli* harboring a 3ABC recombinant plasmid was picked up and grown in 1.5 ml LB broth containing 100 μg/ml of ampicillin and incubated with shaking at 37 ºC for an overnight. The recombinant plasmid was prepared by the QIAgen plasmid miniprep kit (Qiagen, Germany).

3. Sequencing and analysis of 3ABC nucleotide of FMDV type O

The single clone of transformant was analyzed for the nucleotide sequencing. The obtained sequence was compared with the previously reported 3ABC sequence's databases in Genbank as follow; O1Campos (Genbank accession number is AJ320488), Tibet/CHA/99 (Genbank accession number is AJ539138), O1manisa iso87 (Genbank accession number is AY593823), UK2001 iso84 (Genbank accession number is AY593836) and Taiwan97 iso106/112 (Genbank accession number is AY593835); respectively using Bioedit Sequence Alignment Editor (Hall, 1999) and the phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.1 (Tamura et al., 2007) software. The homological data was analyzed.

CHAPTER IV

RESULTS

1. Confirmation of FMDV type O (O-1C$_{124}$ cDNA) by using RT-PCR

The report from Regional Reference Laboratory for Foot and Mouth Disease in South East Asia (RRL) at Nakhon Ratchasima about the samples from the studied farms was positive for FMDV type O by using cell culture technique.

In addition, the author succeeded to extraction for total viral RNA, synthesized the FMDV cDNA, and amplified the O-1C$_{124}$ genes of the five samples from pig farms in Nakhon pathom, Phetchaburi, Ratchaburi provinces and the sample from killed FMDV vaccine.

The PCR amplification products for FMDV type O (O-1C$_{124}$ genes) was run on 2% agarose gel at 110 volts for 60 min. The product lengths for each sample were confirmed and shown at the molecular weight of 1301 bp (Figure 4.1).

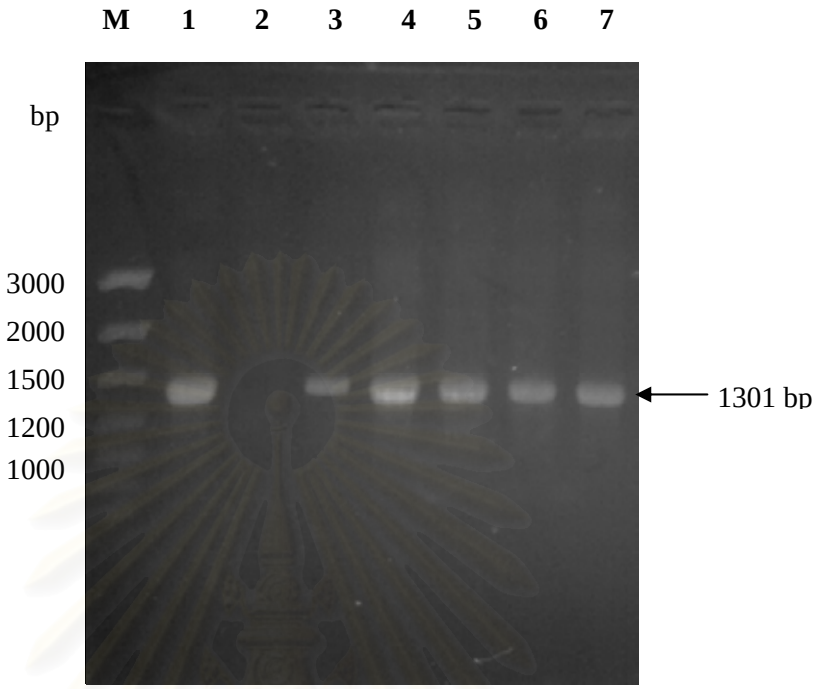


Figure 4.1 Typical agarose gel electrophoresis pattern of the RT-PCR reaction products of FMDV type O (O-1C_{124} genes).

Lane M, 100 bp plus marker;

Lane 1, the positive control from killed vaccine;

Lane 2, the negative control (distilled water);

Lane 3, the sample from Nakhon pathom province;

Lane 4-5, the samples from Phetchaburi province;

Lane 6-7, the samples from Ratchaburi province.

2. The cloning and sequencing of non-structural protein 3ABC gene of FMDV type O

2.1 Amplification of 3ABC cDNA of FMDV type O by using RT-PCR

The PCR amplification products for 3ABC gene of FMDV type O specific from pig farm in Ratchaburi province was run on 2% agarose gel at 110 volts for 60 min. The products lengths for each sample were confirmed and shown at the molecular weight of 1303 bp as shown in Figure 4.2

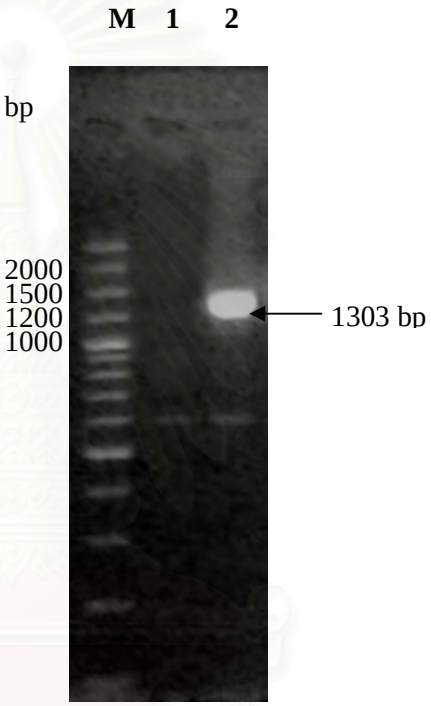


Figure 4.2 2 % agarose gel electrophoresis pattern of 3ABC gene of FMDV type O RT-PCR products.
Lane M, 100 bp plus marker;
Lane 1, the negative control (distilled water).
Lane 2, the sample from Ratchaburi province.

2.2 Cloning of 3ABC cDNA into pET160/GW/D-TOPO vector

The 1303 bp PCR products from 3ABC gene cDNA FMDV type O from pig farm in Ratchaburi province were cut and purified from 2% agarose gel. The purified PCR product was ligated and transformed into *E. coli* strain One Shot®TOP10 (Invitrogen, USA). Ten single white colonies were picked up and the corrected insertion was confirmed by using PCR with specific primers for T7 promoter gene (forward primer) and 3ABC gene (reword primer). In this study, five out of ten colonies of transformants were successfully confirmed and named as pET160/GW/D-TOPO/3ABC-CUVetmed01–05. The expected product sizes for this confirmation were shown at the molecular weight of 1524 bp as shown in Figure 4.3. It was appeared suggested that the selected clone contained a 3ABC expression plasmid with 1303 bp of 3ABC gene insertion. In addition, the five corrected recombinant plasmids were further confirmed by sequencing.

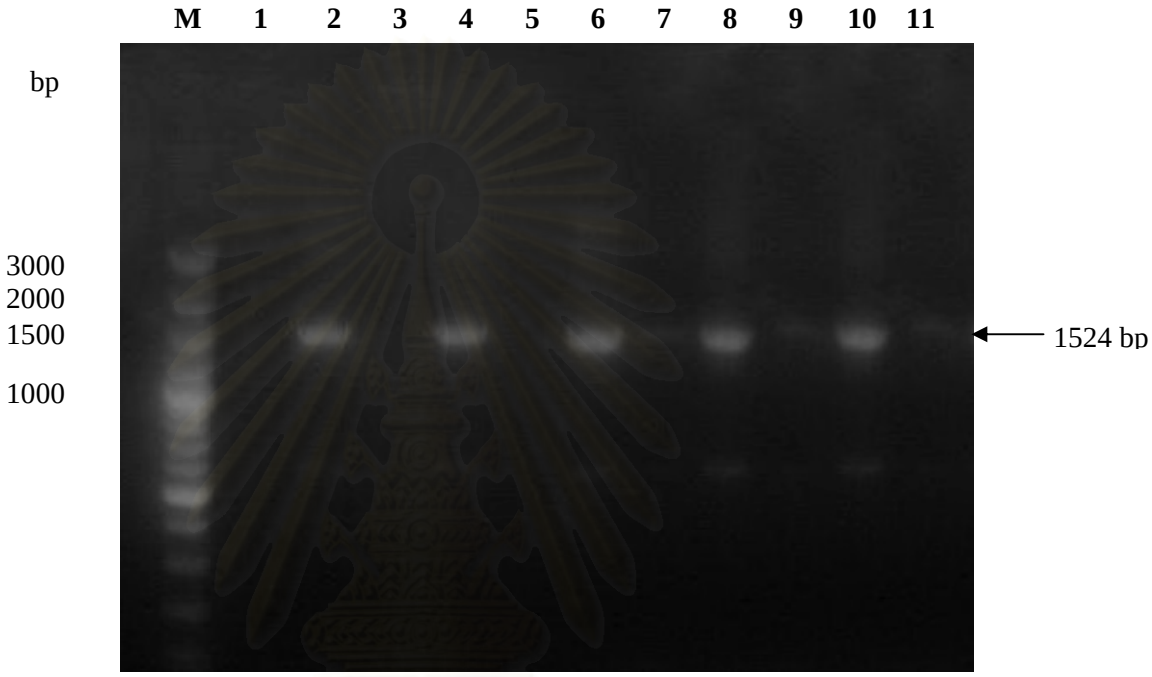


Figure 4.3 Typical agarose gel electrophoresis pattern of the extraction of recombinant PET160/GW/D-TOPO/3ABC-CUVetmed01-05 plasmid with the desired T7 promoter and 3ABC gene primers.

Lane M, 100 bp plus marker;

Lane 1, the negative control (PET160/GW/D-TOPO vector).

Lane 2, 4, 6, 8, and 10, the pET160/GW/D-TOPO/3ABC-CUVetmed01 – 05 plasmids;

Lane 3, 5, 7, 9, and 11, the negative colonies of plasmid DNA.

2.3 Sequencing and analysis of 3ABC nucleotides

The details of five recombinant plasmids of pET160/GW/D-TOPO/3ABC-CUVetmed01 – 05 were successfully sequenced and shown in Table 4.1. The 3ABC nucleotides of five recombinant plasmids were shown similar pattern. The author selected the pET160/GW/D-TOPO/3ABC-CUVetmed01 to further compare with the previously reported 3ABC databases from Genbank as follow; FMDV O1Campos (Genbank accession number is AJ320488), FMDV O1manisa iso87 (Genbank accession number is AY593823), FMDV Taiwan97 iso106/112 (Genbank accession number is AY593835), FMDV Tibet/CHA/99 (Genbank accession number is AJ539138), and FMDV UK2001 iso84 (Genbank accession number is AY593836) by using Bioedit Sequence Alignment Editor (Hall, 1999) software as shown in Figure 4.4. The homology among the pET160/GW/D-TOPO/3ABC-CUvetmed001 and the previously reported 3ABC databases from Genbank were performed the phylogenic tree by using MEGA version 4.1 (Tamura et al., 2007) software (Figure 4.5). The percentage homology between the pET160/GW/D-TOPO/3ABC-CUvetmed001 recombinant plasmids and the previously reported 3ABC databases from Genbank was shown in Table 4.2. The results revealed high percent homology (more than 90%) within this 3ABC region with FMDV O1Campos (90.90 %), FMDV O1manisa iso87 (91.06 %), FMDV Tibet /CHA/99 (100 %) and FMDV UK2001 iso84 (98.93 %). However, the homological data with 3ABC gene of FMDV type O from Taiwan97 iso106/112 was only 82.89%.

The phylogenetic tree of the pET160/GW/D-TOPO/3ABC-CUVetmed01 was compared with the five previously reported 3ABC databases as showed in Figure 4.5. The results showed that the 3ABC gene of FMDV type O from infected pig in Ratchaburi province in 2005 belonged to a group with the FMDV Tibet /CHA/99 and the FMDV UK2001 iso84. However, the FMDV O1Campos, FMDV O1manisa iso87 and FMDV Taiwan97 iso106/112 were grouped apart from those.

Table 4.1 The sequences of 1524 bp of pET160/GW/D-TOPO/3ABC-CUVetmed01-05 recombinant plasmids.

Transformant's name	Sequence
pET160/GW/D-TOPO/3ABC-CUVetmed01	caattccttcccaaaaggctgtgctgtactttctcattgagaagggtcagcacgaagcagcaattga attctttgaggggatggtgcatgactccatcaaggaggagctccggcctctcatccaacagacctc atttgtgaagcgcgcttttaagcgcctgaaggaaaactttgagatagttgccctgtgtttgactcttttg gcaaacatagtgatcatgatccgcgagactcgcaagagacagcagatggtggatgatgcagtga acgagtacattgagaaggcaaacatcaccacggatgacaagactcttgacgaggcggaaaaga accctctggagaccagcggtgccgccactgttggtttcagagagaaaactctcccgggacacaa ggcgagtgatgacgtgaactccgagcccgccaaacccgtggaagaacaaccacaagctgaag gaccctacaccggtccactcgagcgtcaaaaacctctgaaagtgagagccaagctcccacagca ggaggggccctacgctggtccgatggagagacagaaaccgctgaaagtgaaagtgaaagccc cggtcgttaaggaaggaccttacgaaggaccggtgaagaaacctgtcgctttgaaagtgaaagca aagaacttgattgtcactgagagtggtgcyccccccgactgacttgcaaaagatggtcatgggtaac accaagcctgttgagctcatcctcgacgggaagacggtggccatctgctgcgccaccggagtgtt tggtactgcctaccttgttcctcgtcatcttttcgcagagaagtatgacaagatcatgttggacggca gagccatgacagacagtgactacagagtgtttgagtttgagattaaagtgaaaggacaggacatg ctctcagacgccgcgctcatggtgcttcaccgtgggaatcgcgtgcgggacatcacgaagcactt ccgtgatgtggcaagaatgaagaaaggcacccccgtcgtcggcgtgatcaacaacgctgatgttg ggagactgatcttctctggtgaggcccttacctacaaggacattgtagtgtgcatggacggagaca ccatgcccggtctcttcgcctacaaagccgccaccaaggcgggttactgtggaggagccgttctt gcaaaggacggagccgagactttcatcgtcggcactcactccgcaggcggcaacggagttggat actgctcatgcgtttccaggtctatgctgcttaaaatgaaggcacacatcgatcccgaaccacacca cgag
pET160/GW/D-TOPO/3ABC-CUVetmed02	caattccttcccaaaaggctgtgctgtactttctcattgagaagggtcagcacgaagcagcaattga attctttgaggggatggtgcatgactccatcaaggaggagctccggcctctcatccaacagacctc atttgtgaagcgcgcttttaagcgcctgaaggaaaactttgagatagttgccctgtgtttgactcttttg gcaaacatagtgatcatgatccgcgagactcgcaagagacagcagatggtggatgatgcagtga acgagtacattgagaaggcaaacatcaccacggatgacaagactcttgacgaggcggaaaaga accctctggagaccagcggtgccgccactgttggtttcagagagaaaactctcccgggacacaa ggcgagtgatgacgtgaactccgagcccgccaaacccgtggaagaacaaccacaagctgaag gaccctacaccggtccactcgagcgtcaaaaacctctgaaagtgagagccaagctcccacagca ggaggggccctacgctggtccgatggagagacagaaaccgctgaaagtgaaagtgaaagccc

Transformant's name	Sequence
pET160/GW/D-TOPO/3ABC-CUVetmed02	cggtcgttaaggaaggaccttacgaaggaccggtgaagaaacctgtcgctttgaaagtgaaagcaagaacttgattgtcactgagagtggtgcyccccgactgacttgcaaaagatggtcatgggtaaccaccaagcctgttgagctcatcctcgacgggaagacggtggccatctgctgcgccaccggagtgttggtactgcctaccttgttcctcgtcatcttttcgcagagaagtatgacaagatcatgttggacggcagagccatgacagacagtgactacagagtgtttgagtttgagattaaagtgaaaggacaggacatgctctcagacgccgcgctcatggtgcttcaccgtgggaatcgcgtgcgggacatcacgaagcacttccgtgatgtggcaagaatgaagaaaggcacccccgtcgtcggcgtgatcaacaacgctgatgttgggagactgatcttctctggtgaggcccttacctacaaggacattgtagtgtgcatggacggagacaccatgcccggtctcttcgcctacaaagccgccaccaaggcgggttactgtggaggagccgttcttgcaaaggacggagccgagactttcatcgtcggcactcactccgcaggcggcaacggagttggatactgctcatgcgtttccaggtctatgctgcttaaaatgaaggcacacatcgatcccgaaccacaccacgag
pET160/GW/D-TOPO/3ABC-CUVetmed03	caattccttcccaaaaggctgtgctgtactttctcattgagaagggtcagcacgaagcagcaattgaattctttgaggggatggtgcatgactccatcaaggaggagctccggcctctcatccaacagacctcatttgtgaagcgcgcttttaagcgcctgaaggaaaactttgagatagttgccctgtgtttgactcttttggcaaacatagtgatcatgatccgcgagactcgcaagagacagcagatggtggatgatgcagtgaacgagtacattgagaaggcaaacatcaccacggatgacaagactcttgacgaggcggaaaagaaccctctggagaccagcggtgccgccactgttggtttcagagagaaaactctcccgggacacaaggcgagtgatgacgtgaactccgagcccgccaaacccgtggaagaacaaccacaagctgaaggaccctacaccggtccactcgagcgtcaaaaacctctgaaagtgagagccaagctcccacagcaggaggggccctacgctggtccgatggagagacagaaaccgctgaaagtgaaagtgaaagcccccggtcgttaaggaaggaccttacgaaggaccggtgaagaaacctgtcgctttgaaagtgaaagcaaagaacttgattgtcactgagagtggtgcyccccgactgacttgcaaaagatggtcatgggtaaccaccaagcctgttgagctcatcctcgacgggaagacggtggccatctgctgcgccaccggagtgttggtactgcctaccttgttcctcgtcatcttttcgcagagaagtatgacaagatcatgttggacggcagagccatgacagacagtgactacagagtgtttgagtttgagattaaagtgaaaggacaggacatgctctcagacgccgcgctcatggtgcttcaccgtgggaatcgcgtgcgggacatcacgaagcacttccgtgatgtggcaagaatgaagaaaggcacccccgtcgtcggcgtgatcaacaacgctgatgttgggagactgatcttctctggtgaggcccttacctacaaggacattgtagtgtgcatggacggagacaccatgcccggtctcttcgcctacaaagccgccaccaaggcgggttactgtggaggagccgttcttgcaaaggacggagccgagactttcatcgtcggcactcactccgcaggcggcaacggagttggatactgctcatgcgtttccaggtctatgctgcttaaaatgaaggcacacatcgatcccgaaccacaccacgag

Transformant's name	Sequence
pET160/GW/D-TOPO/3ABC-CUVetmed04	caattccttcccaaaaggctgtgctgtactttctcattgagaagggtcagcacgaagcagcaattga attctttgaggggatggtgcatgactccatcaaggaggagctccggcctctcatccaacagacctc atttgtgaagcgcgcttttaagcgcctgaaggaaaactttgagatagttgccctgtgtttgactcttttg gcaaacatagtgatcatgatccgcgagactcgcaagagacagcagatggtggatgatgcagtga acgagtacattgagaaggcaaacatcaccacggatgacaagactcttgacgaggcggaaaaga accctctggagaccagcggtgccgccactgttggtttcagagagaaaactctcccgggacacaa ggcgagtgatgacgtgaactccgagcccgccaaacccgtggaagaacaaccacaagctgaag gaccctacaccggtccactcgagcgtcaaaaacctctgaaagtgagagccaagctcccacagca ggaggggccctacgctggtccgatggagagacagaaaccgctgaaagtgaaagtgaaagccc cggtcgttaaggaaggaccttacgaaggaccggtgaagaaacctgtcgctttgaaagtgaaagca aagaacttgattgtcactgagagtggtgcyccccccgactgacttgcaaaagatggtcatgggtaac accaagcctgttgagctcatcctcgacgggaagacggtggccatctgctgcgccaccggagtgtt tggtactgcctaccttgttcctcgtcatcttttcgcagagaagtatgacaagatcatgttggacggca gagccatgacagacagtgactacagagtgtttgagtttgagattaaagtgaaaggacaggacatg ctctcagacgccgcgctcatggtgcttcaccgtgggaatcgcgtgcgggacatcacgaagcactt ccgtgatgtggcaagaatgaagaaaggcacccccgtcgtcggcgtgatcaacaacgctgatgttg ggagactgatcttctctggtgaggcccttacctacaaggacattgtagtgtgcatggacggagaca ccatgcccggtctcttcgcctacaaagccgccaccaaggcgggttactgtggaggagccgttctt gcaaaggacggagccgagactttcatcgtcggcactcactccgcaggcggcaacggagttggat actgctcatgcgtttccaggtctatgctgcttaaaatgaaggcacacatcgatcccgaaccacacca cgag
pET160/GW/D-TOPO/3ABC-CUVetmed05	caattccttcccaaaaggctgtgctgtactttctcattgagaagggtcagcacgaagcagcaattga attctttgaggggatggtgcatgactccatcaaggaggagctccggcctctcatccaacagacctc atttgtgaagcgcgcttttaagcgcctgaaggaaaactttgagatagttgccctgtgtttgactcttttg gcaaacatagtgatcatgatccgcgagactcgcaagagacagcagatggtggatgatgcagtga acgagtacattgagaaggcaaacatcaccacggatgacaagactcttgacgaggcggaaaaga accctctggagaccagcggtgccgccactgttggtttcagagagaaaactctcccgggacacaa ggcgagtgatgacgtgaactccgagcccgccaaacccgtggaagaacaaccacaagctgaag gaccctacaccggtccactcgagcgtcaaaaacctctgaaagtgagagccaagctcccacagca ggaggggccctacgctggtccgatggagagacagaaaccgctgaaagtgaaagtgaaagccc cggtcgttaaggaaggaccttacgaaggaccggtgaagaaacctgtcgctttgaaagtgaaagca aagaacttgattgtcactgagagtggtgcyccccccgactgacttgcaaaagatggtcatgggtaac accaagcctgttgagctcatcctcgacgggaagacggtggccatctgctgcgccaccggagtgtt

Transformant's name	Sequence
pET160/GW/D-TOPO/3ABC-CUVetmed05	tggtactgcctaccttgttcctcgtcatcttttcgcagagaagtatgacaagatcatgttggacggca gagccatgacagacagtgactacagagtgtttgagtttgagattaaagtgaaaggacaggacatg ctctcagacgccgcgctcatggtgcttcaccgtgggaatcgcgtgcgggacatcacgaagcactt ccgtgatgtggcaagaatgaagaaaggcacccccgtcgtcggcgtgatcaacaacgctgatgttg ggagactgatcttctctggtgaggcccttacctacaaggacattgtagtgtgcatggacggagaca ccatgcccggtctcttcgcctacaaagccgccaccaaggcgggttactgtggaggagccgttctt gcaaaggacggagccgagactttcatcgtcggcactcactccgcaggcggcaacggagttggat actgctcatgcgtttccaggtctatgctgcttaaaatgaaggcacacatcgatcccgaaccacacca cgag

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
3ABCCUVET       caattccttc ccaaaaggct gtgctgtact ttctcattga gaagggtcag
O1Campos        caattccttc ccaaaaatct gtgttgtact ttctcattga gaagggccaa
O1manisa        caattccttc ccaaaagtct gtgttgtact tcctcattga gaaaggccaa
Taiwan97        caatcccttc ccagaagtcc gtgttgtact tcctcattga gaagggccag
Tibet/CHA       caattccttc ccaaaaggct gtgctgtact ttctcattga gaagggtcag
UK2001          caactccttc ccaaaaggct gtgctgtact ttctcattga gaagggccag
                **** ***** *** **  *  *** ****** * ******** *** ** **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
3ABCCUVET       cacgaagcag caattgaatt ctttgagggg atggtgcatg actccatcaa
O1Campos        catgaggcag caattgaatt ctttgagggc atggtccacg actccatcaa
O1manisa        cacgaagcag caattgaatt ctttgaggga atggtgcatg actccatcaa
Taiwan97        cacgaagcag cgatcgagtt cttcgagggg atggtccacg attccatcaa
Tibet/CHA       cacgaagcag caattgaatt ctttgagggg atggtgcatg actccatcaa
UK2001          cacgaagcag caattgagtt ctttgagggg atggtgcatg actccatcaa
                ** ** **** * ** ** ** *** *****  ***** ** * * ********

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
3ABCCUVET       ggaggagctc cggcctctca tccaacagac ctcatttgtg aagcgcgctt
O1Campos        agaggaactc cgacccctca tccaacaaac ttcatttgtg aaacgcgctt
O1manisa        ggaagagctc cggcccctca tccaacagac ctcatttgtg aaacgcgctt
Taiwan97        agaggaactc cgacccctca ttcagcagac ctcgttcgta aaacgcgcct
Tibet/CHA       ggaggagctc cggcctctca tccaacagac ctcatttgtg aagcgcgctt
UK2001          ggaggagctc cggcctctca tccaacagac ctcatttgtg aagcgcgctt
                 ** ** *** ** ** **** * ** ** **  ** *****  ** ***** *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
3ABCCUVET       ttaagcgcct gaaggaaaac tttgagatag ttgccctgtg tttgactctt
O1Campos        tcaagcgcct gaaggaaaat tttgagattg ttgctctgtg tttaacactt
O1manisa        ttaagcgcct gaaggaaaac tttgagactg ttgccctgtg tttgactctt
Taiwan97        tcaagcgcct gaaagagaac tttgaagttg tagctctgtg tttgaccctc
Tibet/CHA       ttaagcgcct gaaggaaaac tttgagatag ttgccctgtg tttgactctt
UK2001          ttaagcgcct gaaggaaaac tttgagatag ttgccctgtg tttgactctt
                * ******** *** ** **  *****    * * ** ***** *** ** **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
3ABCCUVET       ttggcaaaca tagtgatcat gatccgcgag actcgcaaga gacagcagat
O1Campos        ttggcaaaca ttgtgatcat gatccgtgag actcgcaaga ggcagaaaat
O1manisa        ttggcaaaca tagtgatcat gatccgcgag actcgcaaga gacaacagat
Taiwan97        ttggcaaaca tagtgattat gctccgccaa gcgcgcaaga ggtaccaatc
Tibet/CHA       ttggcaaaca tagtgatcat gatccgcgag actcgcaaga gacagcagat
UK2001          ttggcaaaca tagtgatcat gatccgcgag actcgcaaga gacagcagat
                ********** * ***** ** ****** ***  * ******* *  *  *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
3ABCCUVET       ggtggatgat gcagtgaacg agtacattga gaaggcaaac atcaccacgg
O1Campos        ggtggatgat gcagtgaatg agtacattga gaaagcaaac atcaccacag
O1manisa        ggtggacgat gcagtgaatg actacattga gaaggcaaac atcaccacag
Taiwan97        ggtggatgac ccactg---  gac------- ---ggc---- ----------
Tibet/CHA       ggtggatgat gcagtgaacg agtacattga gaaggcaaac atcaccacgg
UK2001          ggtggatgat gcagtgaacg agtacattga gaaggcaaac atcaccacgg
                ****** **   ** **                  ***
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
3ABCCUVET       atgacaagac tcttgacgag gcggaaaaga accctctgga gaccagcggt
O1Campos        atgacaagac tcttgatgag gcggagaaga gccctctaga gaccagcggc
O1manisa        atgacaagac tcttgacgag gcggaaaaga accctctaga gaccagcggt
Taiwan97        --gacgtaac tcttggcgac gcggaaaaga accctctgga gacgagtggc
Tibet/CHA       atgacaagac tcttgacgag gcggaaaaga accctctgga gaccagcggt
UK2001          atgacaagac tcttgacgag gcggaaaaga accctctgga gaccagcggt
                  ***   ** *****  *** ***** ****  ****** ** *** ** **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
3ABCCUVET       gccgccactg ttggtttcag agagaaaact ctcccgggac acaaggcgag
O1Campos        gccagcaccg ttggctttag agagagaact ctcccaggtc aaaaggcatg
O1manisa        gccagcacta ttggtttcag agagagaact ctcccggggc acaaggcgag
Taiwan97        gctagcgctg tcggtttccg agagagatcc cccaccgagc aagggacgcg
Tibet/CHA       gccgccactg ttggtttcag agagaaaact ctcccgggac acaaggcgag
UK2001          gccaccactg ttggtttcag agagaaaact ctcccgggac acaaggcggg
                **   * *   * ** **  * ***** * *  * * * *  * *   * *  *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
3ABCCUVET       tgatgacgtg aactccgagc ccgccaaacc cgtggaagaa caaccacaag
O1Campos        cgatgacgtg aactccgagc ctgcccaacc tgttgaggag caaccacaag
O1manisa        cgatgacgtg agcaccgagc ccgccaaacc cgtggaggac cgaccacaag
Taiwan97        cgaagacgcg aacgctgagc ccgtcgtgtt cggtagggaa caaccgcgag
Tibet/CHA       tgatgacgtg aactccgagc ccgccaaacc cgtggaagaa caaccacaag
UK2001          tgatgacgtg aactccgagc ccgccaaacc cgtggaagaa caaccacaag
                 ** **** * * * * **** * * *       *     **  * *** * **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
3ABCCUVET       ctgaaggacc ctacaccggt ccactcgagc gtcaaaaacc tctgaaagtg
O1Campos        ctgaaggacc ctacgccgga ccactcgagc gtcagaaacc tctgaaagtg
O1manisa        ctgaagggcc ctacgccgga ccacttgagc gtcagaaacc tctgagagtg
Taiwan97        ctgaaggacc ctacgctggg ccactcgagc gtcagaaacc tcttaaagtg
Tibet/CHA       ctgaaggacc ctacaccggt ccactcgagc gtcaaaaacc tctgaaagtg
UK2001          ctgaaggacc ctacaccggt ccactcgagc gtcaaaaacc cctgaaagtg
                ******* ** **** * **  ***** **** **** *****  ** * ****

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
3ABCCUVET       agagccaagc tcccacagca ggaggggccc tacgctggtc cgatggagag
O1Campos        agagccaagc tcccacagca ggaggggcct tacgctggcc cgatggagag
O1manisa        aaaaccaagt tgccacaaca ggagggaccc tacgctggcc cgatggatag
Taiwan97        aaagccgagc tgccacaaca ggagggacca tacgccggcc caatggagag
Tibet/CHA       agagccaagc tcccacagca ggaggggccc tacgctggtc cgatggagag
UK2001          agggccaagc tcccacagca ggaggggccc tacgctggtc cgatggagag
                *   ** **  * ***** ** ****** **  ***** ** * ******* **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
3ABCCUVET       acagaaaccg ctgaaagtga aagtgaaagc cccggtcgtt aaggaaggac
O1Campos        acagaaaccg ctaaaagtga aagcaaaagc cccggtcgtg aaggaaggac
O1manisa        acagaaaccg ttgaaagtga gagcaagagc cccggtcgtg aaggagggac
Taiwan97        acagaaaccg ctaaaggtga aagcaaaagc ccccgtcgtg aaggaaggac
Tibet/CHA       acagaaaccg ctgaaagtga aagtgaaagc cccggtcgtt aaggaaggac
UK2001          acagaaaccg ctgaaagtga aagtgaaagc cccggtcgtt aaggaaggac
                **********  * *******  **  * *** *** *****  ***** ****
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
3ABCCUVET       cttacgaagg accggtgaag aaacctgtcg ctttgaaagt gaaagcaaag
O1Campos        cttacgaggg accggtgaag aagcctgtcg ctttgaaggt gaaagctaag
O1manisa        cctacgaggg accggtgaag aagcctgtcg ctttgaaagt gaaagccaag
Taiwan97        cttacgaggg accggtgaag aaacctgtcg ctttgaaagt gaaagcaaag
Tibet/CHA       cttacgaagg accggtgaag aaacctgtcg ctttgaaagt gaaagcaaag
UK2001          cttacgaagg accggtgaag aaacctgtcg ctttgaaagt gaaagcaaag
                * ***** ** ********** ********** ******* ** ****** ***

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
3ABCCUVET       aacttgattg tcactgagag tggtgcgccc ccgactgact tgcaaaagat
O1Campos        aacctgattg tcactgagag tggtgcccca ccgaccgact tgcaaaagat
O1manisa        aacttgattg tcactgagag tggtgcccca ccgaccgact tgcagaagat
Taiwan97        aacttgatag tcactgagag tggtgcgcca ccgaccgact tgcaaaagat
Tibet/CHA       aacttgattg tcactgagag tggtgcgccc ccgactgact tgcaaaagat
UK2001          aacttgattg tcactgagag tggtgctccc ccgactgact tgcaaaagat
                *** **** * ********** ****** **  ***** **** **** *****

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        710        720        730        740        750
3ABCCUVET       ggtcatgggt aacaccaagc ctgttgagct catcctcgac gggaagacgg
O1Campos        ggtcatgggc aacacaaagc ctgttgagct catcctcgac gggaagacag
O1manisa        ggtcatgggc aacactaagc ctgttgagct catcctcgac gggaagacgg
Taiwan97        ggtcatgggc aacactaagc cagtcgagct catcctcgac ggcaagacgg
Tibet/CHA       ggtcatgggt aacaccaagc ctgttgagct catcctcgac gggaagacgg
UK2001          ggtcatgggt aacaccaagc ctgttgagct catcctcgac gggaagacgg
                ********* ***** **** * ** ***** ********** ** *******

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        760        770        780        790        800
3ABCCUVET       tggccatctg ctgcgccacc ggagtgtttg gtactgccta ccttgttcct
O1Campos        tagccatctg ctgcgctact ggagtgtttg gcactgctta cctcgtgcct
O1manisa        tagccatctg ctgtgctacc ggagtgtttg gcactgccta cctcgtacct
Taiwan97        tagccatttg ctgtgctacc ggagtgttcg gcactgccta cctcgtgcct
Tibet/CHA       tggccatctg ctgcgccacc ggagtgtttg gtactgccta ccttgttcct
UK2001          tggccatctg ctgcgccacc ggagtgtttg gtactgccta cctagttcct
                * ***** ** *** ** *** ******** * * ******** *** ** ***
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        810        820        830        840        850
3ABCCUVET       cgtcatcttt tcgcagagaa gtatgacaag atcatgttgg acggcagagc
O1Campos        cgtcacctct tcgcagagaa gtatgacaag atcatgttgg acggcagagc
O1manisa        cgtcacctct tcgcggagaa gtacgacaag ataatgttgg acggtagagc
Taiwan97        cgtcatctct tcgcggaaaa gtacgacaag atcatgttgg acggcagagc
Tibet/CHA       cgtcatcttt tcgcagagaa gtatgacaag atcatgttgg acggcagagc
UK2001          cgtcatcttt tcgcagagaa gtatgacaag atcatgttgg acggcagagc
                ***** ** * **** ** ** *** ****** ** ******* **** *****

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        860        870        880        890        900
3ABCCUVET       catgacagac agtgactaca gagtgtttga gtttgagatt aaagtgaaag
O1Campos        catgacagac agtgactaca gagtgtttga gtttgagatc aaagtaaaag
O1manisa        catgacagac agtgactaca gagtgtttga gtttgagatt aaagtaaaag
Taiwan97        cttgacagac agtgactaca gagtgtttga gtttgagatt aaagtaaaag
Tibet/CHA       catgacagac agtgactaca gagtgtttga gtttgagatt aaagtgaaag
UK2001          catgacagac agtgactaca gagtgtttga gtttgagatt aaagtgaaag
                * ******** ********** ********** *********  **********
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
3ABCCUVET       gacaggacat gctctcagac gccgcgctca tggtgcttca ccgtgggaat
O1Campos        gacaggacat gctctcagac gccgcgctca tggtgctcca ccgtgggaac
O1manisa        gacaggacat gctctcagac gctgcactca tggtgcttca ccgtgggaac
Taiwan97        gacaggacat gctctcagac gccgctctca tggtgttgca ccgtgggaat
Tibet/CHA       gacaggacat gctctcagac gccgcgctca tggtgcttca ccgtgggaat
UK2001          gacaggacat gctctcagac gccgctctca tggtgcttca ccgcgggaat
                ********** ********** ** ** **** ***** * ** *** *****

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       960        970        980        990       1000
3ABCCUVET       cgcgtgcggg acatcacgaa gcacttccgt gatgtg-gca agaatgaaga
O1Campos        cgcgtgaggg acatcacgaa gcactttcgt gac-acagca agaatgaaga
O1manisa        cgcgtgagag acatcacgaa acattttcgt gac-acagca agaatgaaga
Taiwan97        cgcgtgcgtg acatcacgaa acactttcgt gacgt-agcg agaatgaaga
Tibet/CHA       cgcgtgcggg acatcacgaa gcacttccgt gatgtg-gca agaatgaaga
UK2001          cgcgtgcggg acatcacgaa gcacttccgt gatgtg-gca agaatgaaga
                ****** * * **********  ** ** *** **     **  **********

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1010       1020       1030       1040       1050
3ABCCUVET       aaggcacccc cgtcgtcggc gtgatcaaca acgctgatgt tgggagactg
O1Campos        aaggcacccc cgttgtcggt gtgatcaaca acgccgatgt cgggagactg
O1manisa        aaggcacccc cgttgtcggt gtgatcaaca acgccgacgt tgggagactg
Taiwan97        agggaacccc cgtcgtcggt gtgatcaaca atgctgacgt cgggagactc
Tibet/CHA       aaggcacccc cgtcgtcggc gtgatcaaca acgctgatgt tgggagactg
UK2001          aaggcacccc cgtcgtcggc gtgatcaaca acgctgatgt tgggagactg
                * ** ***** *** *****  ********** * ** ** **  ********

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1060       1070       1080       1090       1100
3ABCCUVET       atcttctctg gtgaggccct tacctacaag gacattgtag tgtgcatgga
O1Campos        attttctctg gtgaggccct tacttacaag gacattgtgg tttgcatgga
O1manisa        attttctctg gagaggccct tacctacaaa gacattgtag tgtgcatgga
Taiwan97        atattctctg gtgaagccct tacttacaag gacatcgtcg tgtgtatgga
Tibet/CHA       atcttctctg gtgaggccct tacctacaag gacattgtag tgtgcatgga
UK2001          atcttctctg gtgaggccct tacctacaag gacattgtag tgtgcatgga
                ** ******* * ** ***** *** *****  ***** ** * * ** *****
                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1110       1120       1130       1140       1150
3ABCCUVET       cggagacacc atgcccggtc tcttcgccta caaagccgcc accaaggcgg
O1Campos        cggagacacc atgcctggcc tctttgccta cagagccgcc accaaggctg
O1manisa        tggagacacc atgccgggcc tgtttgccta cagagccgcc accaaggctg
Taiwan97        tggagacacc atgcctgggc tctttgccta cagggcatcc accaaggcag
Tibet/CHA       cggagacacc atgcccggtc tcttcgccta caaagccgcc accaaggcgg
UK2001          cggagacacc atgcccggtc tcttcgccta caaagctgcc accaaggcgg
                 ********* ***** ** * * ** ***** **  **  ** ******** *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1160       1170       1180       1190       1200
3ABCCUVET       gttactgtgg aggagccgtt cttgcaaagg acggagccga gactttcatc
O1Campos        gctactgcgg aggagccgtt cttgccaaag acggagctga cactttcatc
O1manisa        gttactgcgg gggagccgtt ctcgccaagg acggagccga cacattcatc
Taiwan97        gctactgtgg aggagccgtc ctggcaaagg acggggccga aacgttcatc
Tibet/CHA       gttactgtgg aggagccgtt cttgcaaagg acggagccga gactttcatc
UK2001          gttactgtgg aggagccgtt cttgcaaagg acggagccga gactttcatc
                * ***** **  ********* ** ** **** **** ** **  ** ******
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1210       1220       1230       1240       1250
3ABCCUVET       gtcggcactc actccgcagg cggcaacgga gttggatact gctcatgcgt
O1Campos        gtcggcactc actctgcagg aggcaacgga gttggatact gctcatgcgt
O1manisa        gttggcactc actccgcagg tggtaacgga gttggatact gctcgtgcgt
Taiwan97        gttggcaccc actccgcagg tggaaacggc ataggatact gttcgtgtgt
Tibet/CHA       gtcggcactc actccgcagg cggcaacgga gttggatact gctcatgcgt
UK2001          gtcggcactc actccgcagg cggcaatgga gttggatact gctcatgcgt
                ** ***** * **** *****  ** ** **   * ******* * ** ** **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1260       1270       1280       1290       1300
3ABCCUVET       ttccaggtct atgctgctta aaatgaaggc acacatcgat cccgaaccac
O1Campos        ttccaggtcc atgcttctta aaatgaaggc acacattgac cccgaaccac
O1manisa        gtccaggtcc atgctcctga aaatgaaggc acacattgac cctgaaccac
Taiwan97        ttcccgatca atgctcctga agatgaaggc acacatcgac cctgaaccac
Tibet/CHA       ttccaggtct atgctgctta aaatgaaggc acacatcgat cccgaaccac
UK2001          ttccaggtcc atgctgctta aaatgaaggc acacatcgat cccgaaccac
                 *** * **  ***** ** * * ******** ****** **  ** *******

                ....|...

3ABCCUVET       accacgag
O1Campos        accacgag
O1manisa        accacgag
Taiwan97        accacgag
Tibet/CHA       accacgag
UK2001          accacgag
                ********
```

Figure 4.4 The nucleotide aligment of the O1Campos (Genbank accession number is AJ320488), O1manisa iso87 (Genbank accession number is AY593823), Taiwan97 iso106/112 (Genbank accession number is AY593835), Tibet/CHA/99 (Genbank accession number is AJ539138), UK2001 iso84 (Genbank accession number is AY593836), and pET160/GW/D-TOPO/3ABC-CUVetmed01 plasmid; respectively.

Table 4.2 The percentage of the homological data compared between the five 3ABC FMDV type O sequences reported from Genbank and the pET160/GW/D-TOPO/3ABC-CUvetmed01.

	3ABC CUvetmed 01	O1Campos	O1manisa iso87	Taiwan97 iso106/112	Tibet/CHA /99	UK2001 iso84
3ABC CUvetmed01		90.90	91.06	82.89	100.00	98.93
O1Campos	90.90		91.21	83.10	90.90	90.51
O1manisa iso87	91.06	91.21		83.42	91.06	90.67
Taiwan97 iso106/112	82.89	83.10	83.42		82.89	82.81
Tibet/CHA/99	100.00	90.90	91.06	82.89		98.93
UK2001 iso84	98.93	90.51	90.67	82.81	98.93	

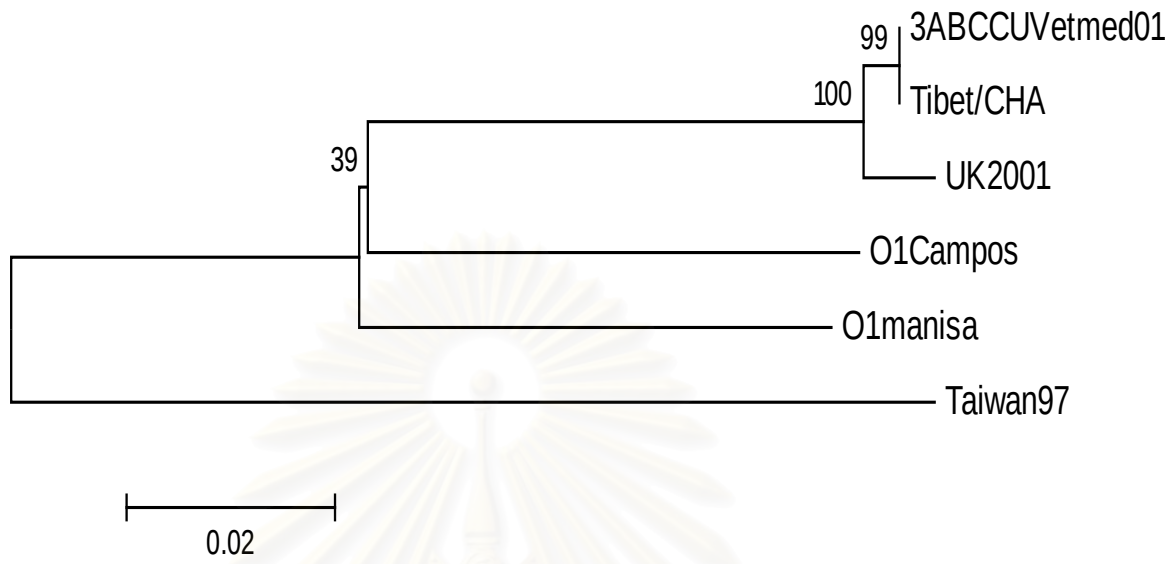


Figure 4.5 The phylogenetic tree of the pET160/GW/D-TOPO/3ABC-CUVetmed01 compared with the five previously reported 3ABC databases.

The tree was generated from nucleotide sequence of 3ABC gene using the neighbor-joining algorithm method. The length of horizontal branches reflects phylogenetic distance relationship. The vertical lines were non-informative and set for clearity only.

CHAPTER V

DISCUSSION

In this study, the author succeeded in the confirmation of FMDV type O from the field sample by using RT-PCR. It may become useful tools for the typing of FMDV type O from the field sample and investigation of FMD in Thailand.

The FMDV type O sample was selected from pig farm in Ratchaburi province. The reason for choosing this sample to amplify 3ABC cDNA and clone into pET160/GW/D-TOPO vector is the first province that officially reported incidence of FMD outbreak in 2005. Furthermore, the local FMDV sample should be more suitable to be an antigen for detection of antibody from FMDV infected pigs in Thailand because of the similarity of immune responses induced by a chosen FMDV sample and by infection from a field virus.

Pigs are important economic animals in Thailand and a vaccination program is usually implemented in Thai pig population with trivalent FMDV killed vaccine made in Thailand. Although, it is considered that vaccinated pigs should not become carriers after field infection, the assay based on antibody against NSP is still important for FMDV eradication program in pig population (Chung et al., 2002). Rodriguez et al. (1994) assessed the immunogenicity of different FMDV proteins in swine. After analyzing specificity of anti-FMDV antibodies produced against NSPs in sera from infected and vaccinated pigs, they concluded that the NSP 3ABC was the most immunogenic virus-induced polypeptide and would be used to distinguish between infected and vaccinated pigs. It was also shown that the NSP 3ABC antibodies were detectable from two weeks post-infection. Other studies have shown that infected animals can be clearly differentiated from vaccinated animals by the presence of antibodies to 2C, 3A and 3ABC induced by viral replication following infection (Mackay et al., 1998). There is considerable variation in the magnitude of the overall response to NSPs in individual animals to each of NSPs examined, which in turn reflected the different extent of viral replication. Therefore, the amount of NSP to which animal was exposed and the difference in the immunogenicity of the different NSPs was observed. However, it was concluded that the NSPs 3ABC was the most reliable single indicator of infection in both bovine and porcine sera (Mackay et al., 1998). The immune response to 3ABC appeared early after infection and antibody to 3ABC could be detected for longer than antibody to other

NSP. Clavijo et al. (2004[b]) studied the detection level of antibody responses to NSPs in animals infected with different serotypes of FMDV. They did not find any variation in the detection.

In this study, RT-PCR was successful for confirmation of FMDV type O and amplification of 3ABC gene cDNA of FMDV type O. Following RT-PCR technique, some mutation may occur due to the lack of error repairing systems in the reverse transcriptase as mentioned before. It has also been suggested that the reverse transcriptase can be responsible for mutations but Cottam et al. (2009) reported no significantly difference between the proof reading reverse transcriptase and no-proof reading reverse transcriptase. However, other conditions should be varied to reduce errors. The oligonucletide primers are the one factor to be concerned and the primer concentration is also importance. The high primer concentration may lead to misprint and accumulation of non-specific PCR products. Generally, five pmol of each primer is an optimal concentration; therefore each primer should be optimized (Kawasaki et al., 1990). In this study, RT-PCR was run following the Access-RT-PCR protocol. However, the concentration of the primers was optimized at twenty pmol which are the lowest effective concentration and the lowest amount of primer dimers.

RT-PCR using total RNA from FMDV type O infected pig in Ratchaburi resulted in band of 1303 bp, corresponding to the complete 3ABC coding sequence excluding 3 and 4 bases at the 5' and 3' ends, respectively. These nucleotide bases were removed to avoid proteolytic cleavage of the 2B-3A junction by the 3C protease. Clavijo et al. (2004[b]) reported protein expression experiments and indicated that if the sense primer included the 3' end coding sequences of the 2B protein, the 3ABC protein was expressed but 6xHis tag would not be detected by western blotting probably due to a proteolytic cleavage of this 6xHis tag.

The 3ABC cDNA from FMDV infected pig in Ratchaburi province in 2005 was cloned into pET160/GW/D-TOPO vector. There are factors concerning in selecting the cloning and expression systems. There were largely depends on the biochemical and biological properties of interested proteins, the requirement of an amount of recombinant proteins, as well as the nature of the experiments (Geisse et al., 1996). The 3ABC cDNA of FMDV was successfully cloned and expressed in many systems and expressed proteins had immunogenicity to react with anti-3ABC antibodies from FMDV infected pigs. In this study, *E. coli* cloning and expression system were selected because this system was cheap, fast and easy to manipulate. Other reports had preferred to produce the 3ABC protein in baculovirus to avoid many problems such as the production of recombinant proteins in an insoluble form or

inclusion body (Silberstein et al., 1997). In addition, the possibility of having false positives due to the presence of anti *E.coli* antibodies. However, recombinant proteins produced from baculovirus was more expensive, required cell culture facilities and usually resulted in a lower yield than *E.coli* derived recombinant proteins (Clavijo et al., 2004[b]).

The plasmid pET160/GW/D-TOPO provides high ligation efficiency due to the attached topoisomerase at both ends of this linear plasmid. The directional ligation of blunt end PCR product saves time for confirming gene orientation, so it needed only PCR technique to determine the inserted gene. In addition, the Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move interested gene into multiple vector systems (Invitrogen, 2004). This system can provide alternative method to transfer the 3ABC gene of FMDV type O to the other expression vectors such as yeast, baculovirus and mammalian system.

Generally, NSPs are crucial roles for viral propagation and are more conserved region than the structural proteins (Feng et al., 2004). The mutations or deletions in the NSPs could be detrimental to viral replication and protein processing (van Rensburg et al., 2002). The VP4, VP2, VP3, 2A, 2B, 2C, 3B, 3C and 3D nucleotides were more conserved among and within the different FMDV strains. The 3A nucleotide exhibited the lowest conservation similarity among Cathay topotype and PanAsia strains of FMDV type O (Zhao et al., 2008). But 3A nucleotide of the FMDV type A Indian isolates was also conserved (Mohapatra et al., 2009).

In this study, the 3ABC of FMDV type O was completely sequenced and reported for the first time. The homology and the generation of phylogenetic tree of this gene in FMDV type O were analyzed. The sequence showed at 82 – 100% homology of 3ABC nucleotides with other previously reports from Genbank databases. This result indicated that 3ABC gene of FMDV type O is the conserved gene and correlated with study from Sun et al. (2004) which showed that the 3ABC nucleotide and amino acid sequences among different strains of FMDV were similar. From sequential analysis, pET160/GW/D-TOPO/3ABC-CUVetmed01 showed 100% similarity at nucleotide level with Tibet/CHA/99 strain. Furthermore, pET160/GW/D-TOPO/3ABC-CUVetmed01 was strongly clustered with UK2001 iso84, suggesting a relatively recent common ancestor for these isolates. Although this virus causing outbreak in Ratchaburi was genetically and most closely related to that causing an outbreak of type O in Tibet, it is not clear and no evidence that viruses were transmitted between Thailand

and Tibet. However, FMDV genome region coding for other regions such as outer-capsid polypeptide, VP1 sequences are necessary to further study and useful for determining the prevalence of intratypic recombination in the filed.

Taken together, the system developed in this study may become useful tools for further improvements of technique for expression of recombinant 3ABC proteins. It will develop specific recombinant proteins for detection antibody to FMDV infected pigs in Thailand. This study also provides a preliminary 3ABC sequence's data of FMDV type O in a Thai pig and allows us to understand more on genotype of FMDV by genetic characterization.

Conclusion

This report represented study of molecular biology of 3ABC gene of FMDV type O in Ratchaburi province in 2005. The confirmation of FMDV type O (O-1C_{124} cDNA) using RT-PCR technique may be beneficial for a monitoring and a diagnostic tool in typing confirmation of FMDV type O in swine herds where epizootic viruses are concerned. Moreover, the author described the cloning, sequencing and construction of plasmid 3ABC gene DNA expression vector in this thesis. The obtained knowledge and tools in this study contributes to not only on the basic research of 3ABC nucleotides of FMDV type O but also on the applied researches for improved diagnostic tools to detect FMDV infection in pig.

From this study, suggestions for further studies are FMDV 3ABC recombinant protein expression, purification and reactivity of this recombinant protein.

REFERENCES

Alexandersen, S., Brotherhood, I. and Donaldson, A.I. 2002. Natural aerosol transmission of foot-and-mouth disease virus to pigs: minimal infectious dose for strain O1 Lausanne. Epidemiol. Infect. 128: 301–312.

Alexanderson , S., Zhang, Z., Donaldson, A.I. and Garland, A.J. 2003. The pathogenesis and diagnosis of foot-and-mouth disease. J. Comp. Pathol. 129: 1–36.

Bachrach, H. L. 1968. Foot and mouth disease. Annu. Rev. Microbiol. 22: 201–244.

Beard, C.W. and Mason, P.W. 2000. Genetic determinants of altered virulence of Taiwanese foot-and-mouth disease virus. J. Virol. 74: 987–991.

Beck, E., Forss, S., Strebel, K., Cattaneo, R. and Feil, G. 1983. Structure of the FMDV translation initiation site and of the structural proteins. Nucleic Acids Res. 11: 7873–7885.

Belsham, G.J. 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. Biophysic Mol. Biol. 60: 241–260.

Berger, H.G., Straub, O.C., Ahl, R., Tesar, M. and Marquardt, O. 1990. Identification of foot-and-mouth disease virus replication in vaccinated cattle by antibodies to non structural virus proteins. Vaccine. 8: 213–216.

Bergmann, I.E., Malirat, V., Neitzert, E., Beck, E., Panizzutti, N., Sanchez, C. and Falczuk, A. 2000. Improvement of a serodiagnostic strategy for foot and mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. Arch. Virol. 145: 473–489.

Brown, C.C., Olander, H.J. and Meyer, R.F. 1995. Pathogenesis of foot and mouth disease in swine, studied by in-situ hybridization. J. Comp. Pathol. 113: 51–58.

Buenz, E.J. and Howe, C.L. 2006. Picornaviruses and cell death. Trend Micro. 14(1): 28-36

Cao, X., Bergmann, I.E., Fullkrug, R. and Beck, E. 1995. Functional analysis of the two alternative translation initiation sites of foot-and-mouth disease virus. J. Virol. 69: 560–563.

Chung, W.B., Sorensen, K.J., Liao, P.C., Yang, P.C. and Jong, M.H. 2002. Differentiation of foot and mouth disease virus-infected from vaccinated pigs by enzyme-linked immunosorbent assay using non-structural protein 3AB as the antigen and application to an eradication program. J. Clin. Microbiol. 40: 2843–2848.

Clarke, B.E., Sangar, D.V., Burroughs, J.N., Newton S.E., Carroll, A.R. and Rowlands, D.J. 1985. Two initiation sites for foot-and-mouth disease virus polyprotein in vivo. J. Gen. Virol. 66: 2615–2626.

Clavijo, A., Wright, P. and Kitching, P. 2004 [a]. Developments in diagnostic techniques for differentiating infection from vaccination in foot and mouth disease. Vet. J. 167(1): 9–22.

Clavijo, A., Zhou, E.M., Hole, K., Galic, B. and Kitching, P. 2004[b]. Development and use of a biotinylated 3ABC recombinant protein in a solid-phase competitive ELISA for the detection of antibodies against foot-and-mouth disease virus. J. Virol. Methods. 120(2): 217–227.

Cottam, E.M., King, D.P., Wilson, A., Paton, D.J. and Haydon, D.T. 2009. Analysis of Foot and mouth disease virus nucleotide sequence variation within naturally infected epithelium. Virus Res. 140: 199-204.

Cowan, K. M. and Graves, J. H. 1966. A third antigenic component associated with foot and mouth disease infection. Virology. 30: 528–540.

Department of Livestock Development. 2004[a]. Incidence of Foot and mouth disease: 2004. Epidemic Survilance Report. 10(special): 2-5.

Department of Livestock Development. 2004[b]. Report of Foot and mouth disease: December 2004. Epidemic Survilance Report. 10(110): 2-5.

Department of Livestock Development. 2005[a]. Incidence of Foot and mouth disease. Epidemic Survilance Report. 13(special): 2-4.

Department of Livestock Development. 2005[b]. Report of Foot and mouth disease: October 2005. Epidemic Survilance Report. 11(120): 10-11.

Department of Livestock Development. 2005[c]. Report of Foot and mouth disease: December 2005. Epidemic Survilance Report. 11(special): 2-5.

Department of Livestock Development. 2006. Incidence of Foot and mouth disease: 2006 Epidemic Survilance Report. 13(122): 10-11.

Diego, M.D., Brocchi, E., Mackay, D. and Simone, F.D. 1997. The non-capsideal polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Arch. Virol. 142: 2021–2033.

Doel, T. 1996. Natural and vaccine-induced immunity to foot and mouth disease: the prospects for improved vaccines. Revue scientifique et technique (International Office of Epizootics) 15: 883–911.

Donnelly, M.L.L., Gani, D., Flint, M., Monaghan, S. and Ryan, M.D. 1997. The cleavage activities of aphthovirus and cardiovirus 2A proteins. J. Gen.Virol. 78: 13–21.

Eble, P.L., Bouma, A., Weerdmeester, K., Stegeman, J.A. and Dekker, A. 2007. Serological and mucosal immune responses after vaccination and infection with FMDV in pigs. Vaccine. 25: 1043–1054.

Feng, Q., Yu, H., Liu, Y., He, C., Hu, J., Sang, H., Ding, N., Ding, M., Fung, D., Lau, L., Yu, C. and Chen, J. 2004. Genome comparison of a novel foot-and-mouth disease virus with other FMDV strains. Biochem. Biophys. Res. Commun. 323(1): 254-263.

Forss, S., Strebel, K., Beck, E. and Schaller, H. 1984. Nucleotide sequence and genome organization of foot and mouth disease virus. Nucleic Acids Res. 12 (16): 6587–6601.

Geisse, S., H. Gram, B. Kleuser and H.P. Kocher. 1996. Eukaryotic expression systems: a Comparison. Protein Express. and Purificat. 8: 271–282.

Grubman, M. J. 1980. The 5’ end of foot-and-mouth disease virion RNA contains a protein covalently linked to the nucleotide pUp. Arch. Virol. 63: 311–315.

Grubman, M. J., Robertson, B. H., Morgan, D. O., Moore, D. M. and Dowbenko, D. 1984. Biochemical map of polypeptides specified by foot and mouth disease virus. J. Virol. 50: 579–586.

Grubman, M.J. and Baxt, B. 2004. Foot and mouth disease. Clin. Microbiol. Reviews. 17(2): 465-493.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for window 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95-96.

Hartley, C.A., Ficorilli, N., Dynon, K., Drummer, H.E., Huang, J., Studdert, M.J. 2001. Equine rhinitis A virus: structural proteins and immune response. Gen Virol 82: 1725–1728.

Hobson, S.D., Rosenblum, E.S., Richards, O.C., Richmond, K., Kirkegaard, K. and Schultz, S.C. 2001. Oligomeric structures of poliovirus polymerase are important for function. EMBO. J. 20: 1153–1163.

Invitrogen. 2004. Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology: Instruction manual.

Kawasaki, E.S. 1990. Amplification of RNA. In: PCR Protocols: A Guide to Methods and Applications. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J.(eds). San Diego: Academic Press. 21-7

Kitching, R.P. 1998. A recent history of foot and mouth disease. J. Comp. Pathol. 118: 89–108.

Kitching, R.P. 2004. Diagnosis and Control of Foot and Mouth Disease. In: Foot and Mouth Disease Current Perspective. Sobrino, F. and Domingo, E. (eds). Norfolk: Horizon Bioscience. 417-418.

Kitching, R.P. and Alexandersen, S. 2002. Clinical variation in foot and mouth disease: pigs. Rev. Sci. Tech. Off. Int. Epizoot. 21: 513–518.

Kitching, R.P., Hutber, A.M. and Thrusfield, M.V. 2005. A review of foot and mouth disease with special consideration for the clinical and epidemiological factors relevant to predictive modelling of the disease. Vet. J. 169(2): 197-209.

Kitson, J.D., McCahon, D. and Belsham, G.J. 1990. Sequence analysis of monoclonal antibody resistant mutants of type O foot and mouth disease virus: evidence for the involvement of the three surface exposed capsid protein in four antigenic sites. Virology. 179(1): 26-34.

Klump, W., Marquardt, O. and Hofschneider, P. H. 1984. Biologically active protease of foot and mouth disease virus is expressed from cloned viral cDNA in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 81: 3351–3355.

Knowles, N.J. and Samual, A.R. 1994. Polymerase chain reaction amplification and cycle sequencing of the 1D (VP1) gene of foot and mouth disease virus. Proceedings of Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth disease. Vienna. 45-53.

Knowles, N.J., Davies, P.R., Henry, T., Donnell, V.O., Pacheco, J.M. and Mason, P.W. 2001. Emergence in Asia of foot-and-mouth disease viruses with altered host range: characterization of alterations in the 3A protein. J. Virol. 75: 1551–1556.

Knowles, N.J., Samual, A.R., Davies, P.R., Midgley, R.J. and Valarcher, J.F. 2005. Pandemic strain of foot and mouth disease virus serotype O. Emerging Infect. Dis. 11: 1887–1893.

Kweon, C.H., Ko, Y.J., Kim, B.J., Kwon, J.H., Hyun, B.H., Kang, S.W., Joo, Y.S. and Lubroth, J. 2002. Molecular characterization of foot-and-mouth disease virus O/SKR/2000. Virus. Res. 90: 15-22.

Kweon, C.H., Ko, Y.J., Kim, W.I., Lee, S.Y., Nah, J.J., Lee, K.N., Sohn, S.J., Choi, K.S., Hyun, B.H., Kang, S.W., Joo, Y.S. and Lubroth, J. 2003. Development of a foot and mouth disease NSP ELISA and its comparison with differential diagnostic methods. Vaccine. 21(13-14): 1409-1414.

La Torre, J.L., Grubman, M.J., Baxt, B. and Bachrach, H.L. 1980. The structural polypeptides of aphthovirus are phosphoproteins. Proceeding of National Academy of Sciences. United States of America. December: 7444-7447.

Linchongsubongkoch, W. 2003. Recent characteristic of FMD virus in Thailand. Proceeding of The International Symposium of The World Association of the World Association of Veterinary Laboratory Diagnosticians and OIE Seminar on Biotechnology. Bangkok. November 9-13: s9-s15.

López, M.G., Peralta, A., Berinstein, A., Fondevila, N., Carrillo, E. and Taboga, O. 2005. High-level expression of recombinant 3AB1 non-structural protein from FMDV in insect larvae. J. Virol. Methods. 124(1-2): 221-224.

Lowe, P.A. and Brown, F. 1981. Isolation of a soluble and template dependent foot and mouth disease virus RNA polymerase. Virology. 111: 23–32.

Lubroth, J., Grubman, M.J., Burrage, T.G., Newman, J.F.E. and Brown, F. 1996. Absence of protein 2C from clarified foot-and-mouth disease virus vaccines provides the basis from distinguishing convalescent from vaccinated animals. Vaccine. 5: 419–426.

Lubroth, J. and Brown, F. 1995. Identification of native foot-and-mouth disease virus nonstructural protein 2C as a serological indicator to differentiate infected from vaccinated livestock. Res. Vet. Sci. 59: 70–78.

Lu, Z., Cao, Y., Guo, J., Qi, S., Li, D., Zhang, Q., Ma, J., Chang, H., Liu, Z., Liu, X. and Xie, Q. 2007. Development and validation of a 3ABC indirect ELISA for differentiation of foot-and-mouth disease virus infected from vaccinated animals. Vet. Microbiol. 125(1-2): 157-169.

Mackay, D.K.J., Forsyth, M.A., Davies, P.R., Berlinzani, A., Belsham, G.J., Flint, M. and Ryan, M.D. 1998. Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. Vaccine. 16(5): 446-459.

Malirat, V., Neitzer, E., Bergmann, I.E., Maradei, E. and Beck, E. 1998. Detection of cattle exposed to foot-and-mouth disease virus by means of an indirect ELISA test using bioengineered nonstructural polyprotein 3ABC. Vet. Q. 20: S24–S26.

Mateu, M.G. 1995. Antibody recognition of picornaviruses and escape from neutralization: a structural view. Virus Res. 38(1): 1-24.

Meyer, R.F., Babcock, G.D., Newman, F.J., Burrage, T.G., Toohey, K., Lubroth, J. and Brown, F. 1997. Baculovirus expressed 2C of foot and mouth disease virus has the potential for differentiating convalescent from vaccinated animals. J. Virol. Methods. 65: 33-43.

Mohapatra, J.K., Sahu, A., Pandey, L., Sanyal, A., Hemadri, D. and Pattnaik, B. 2009. Genetic characterization of type A foot and mouth disease virus 3A region in context of the reemergence of VP3^{59}-deletion lineage in India. Infect. Gent. Evol. doi: 10.1016/j.meegid.2009.01.009.

Newman, J.F., Cartwright, B., Doel, T.R. and Brown, F. 1979. Purification and identification of the RNA-dependent RNA polymerase of foot and mouth disease virus. J. Gen. Virol. 45: 497–507.

Newman, J.F.E., Piatti, P.G., Gorman, B.M., Burrage, T.G., Ryan, M.D., Flint, M. and Brown, F. 1994. Foot-and-mouth disease virus particles contain replicase protein 3D. Microbiol. 91: 733-737.

Office International des Epizooties. 2005. Annual OIE/FAO FMD Reference Laboratory Network Report. Paris, France. 38.

Official of Agricultural commodity and food standard. 2004. Thai agricultural commodity and food Standard TACFS 10400 - 2004: Diagnostic test of foot and mouth disease. Bangkok, Thailand. 17.

Oleksiewicz, M.B., Donaldson, A.I. and Alexandersen, S. 2001. Development of a novel real-time RT-PCR assay for quantitation of foot-and mouth disease virus in diverse porcine tissues. J. Virol. Methods. 92: 23–35.

Paarlberg, P.L., Lee, J.G., and Seitzinger, A.H. 2002. Potential revenue impact of an outbreak of foot and mouth disease in the United States. J. Am. Vet. Med. Assoc. 220: 988–992.

Piccone, M.E., Rieder, E., Mason P.W. and Grubman, M.J. 1995. The foot and mouth disease virus leader proteinase gene is not required for viral replication. J. Virol. 69: 5376–5382.

Reid, S.M., Ferris, N.P., Hutchings, G.H., Clercq, K.D., Newman, B. J., Knowles, N.J., and Samual, A.R. 2001. Diagnosis of foot-and-mouth disease by RT-PCR: use of phylogenetic data to evaluate primers for the typing of viral RNA in clinical samples. Arch. Virol. 146: 2421–2434.

Remond, M., Kaiser, C. and Lebreton, F. 2002. Diagnosis and screening of foot and mouth disease. Comp. Immunol. Microbiol. Infect. Dis. 25(5-6): 309-320.

Rigden, R.C., Carrasco, C.P., Summerfield, A. and McCullough, K.C. 2002. Macrophage phagocytosis of foot-and-mouth disease virus may create infectious carriers. Immunology. 106: 537–548.

Roberts, P.J. and Belsham, G.J. 1995. Identification of critical amino acids within the foot-and-mouth disease virus leader protein, a cysteine protease. Virology. 213: 140–146.

Robertson, B.H., Morgan, D.O., Moore, D.M., Grubman, M.J., Card, J., Fischer, T., Weddell, G., Dowbenko, D. and Yansura, D., Small, B. and Kleid, D.G. 1985. Nucleotide and amino acid sequence coding for polypeptides of foot and mouth disease virus type A12. J. Virol. 54:651–660.

Rodriguez, A., Dopazo, J., Saiz, J.C. and Sobrino, F. 1994. Immunogenicity of non-structural proteins of foot-and-mouth disease virus: differences between infected and vaccinated swine. Arch. Virol. 136: 123–131.

Rowlands, D.J. 2004. Foot and Mouth Disease Virus Peptide Vaccines. In: Foot and Mouth Disease Current Perspective. Sobrino, F. and Domingo, E. (eds). Norfolk: Horizon Bioscience. 338.

Rueckert, R.R., and Wimmer, E. 1984. Systematic nomenclature of picornavirus proteins. J. Virol. 50:957–959.

Sangar, D.V., Newton, S.E., Rowlands, D.J. and Clarke, B.E. 1987. All foot and mouth disease virus serotypes initiate protein synthesis at two separate AUGs. Nucleic Acids Res. 15: 3305–3315.

Santos, T.L., Wu, Q., Botton, S.A. and Grubman, M.J., 2005. Short hairpin RNA targeted to the highly conserved 2B nonstructural protein coding region inhibits replication of multiple serotypes of foot-and-mouth disease virus. Virology. 335: 222-231.

Sanz-Parra, A., Sobrino, F. and Ley, V. 1998. Infection with foot and mouth disease virus results in a rapid reduction of MHC class surface expression. J. Gen. Virol. 79: 433–436.

Silberstein, E., Kaplan, G., Taboga, O., Duffy, S. and Palma, E. 1997. Foot and mouth disease virus infected but not vaccinated cattle develop antibodies against recombinant 3AB1 nonstructural protein. Arch. Virol. 142**:** 795–805.

Shen, F., Chen, P.D., Walfield, A.M., Ye, J., House, J., Brown, F. and Wang, C.Y. 1999. Differentiation of convalescent animals from those vaccinated against foot and mouth disease by a peptide ELISA. Vaccine. 17: 3039–3049.

Sorensen, K.J., Madsen, K.G., Madsen, E.S., Salt, J.S., Nqindi, J. and Mackay, D.K.J. 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Arch. Virol. 143: 1461–1476.

Strebel, K., and Beck, E. 1986. A second protease of foot and mouth disease virus. J. Virol. 58: 893–899.

Sun, T., Lu, P. and Wang, X. 2004. Localization of infection-related epitopes on the non structural protein 3ABC of foot and mouth disease virus and the application of tandem epitopes. J. Virol. Methods 119: 79-86.

Tamura, K., Dudley, J., Nei, M. And Kumar, S. 2007. MEGA 4.1: Molecular Evolutionary Genetics Analysis (MEGA) Software version 4.1. Mol. Biol. Evo. 24: 1596-1599.

Turner, P., McLennan, A., Bates, A. and White, M. 2005. Analysis and Uses of Cloned DNA. In: Molecular Biology. 3th ed. Abingdon: Taylor and Francis Group. 180-181.

Thongtha, P. and Linchongsubongkoch, W. 2006. Molecular Epidemiology Analysis of Foot and Mouth Disease Virus Type A Field Outbreaks in Thailand During 2004-2005. National Institue of Animal Health J. 3(1): 18-27.

Vakharia, V.N., Devaney, M.A., Moore, D.M., Dunn, J.J. and Grubman, M.J. 1987. Proteolytic processing of foot and mouth disease virus polyproteins expressed in a cell-free system from clone-derived transcripts. J. Virol. 61: 3199–3207.

van Rensburg, H., Haydon, D., Joubert, F., Bastos, A. Health, L., Nel, L. 2002. Genetic heterogenicity in the foot and mouth disease virus leader and 3C proteinase. Gene. 289: 19-29.

Wimmer, E. 1982. Genome-linked proteins of viruses. Cell. 28: 199–201.

Zhao, M., Suo, Q., Chen, J., Chen, L. And Xu, Y. 2008. Sequence analysis of the protein-coding regions of foot and mouth disease virus O/HK/2001. Vet. Microl. 130: 238-246.

APPENDICES

APPENDIX A

Instruments and chemical substances

1. A -20°C refrigerator, Model SF-C997 (Sanyo, Thailand)
2. A -80°C refrigerator, Model 905 (Thermofisher Scientific, USA.)
3. Centrifuge and Microcentrifuge
4. Experimental glasswares
5. Gel document system, Model GVM 20 (Synoptics, UK.)
6. Gel electrophoresis system, Model GE-100 (Bioer technology co. Ltd., China)
7. Heat block (Labnet International Inc., USA)
8. Incubator, Model BE-400 (Memmert Inc., Germany)
9. Lamina air flow, Model Bio II A (Telstar, Spain)
10. Micropipette (Labnet, USA.) and Micropipette tips
11. PCR assay
    - 11.1 Agarose gel (Molecular grade)
    - 11.2 100 bp plus DNA marker (Generuler™, Fermentus, Canada)
    - 11.3 1 kbp DNA marker (Promega, USA)
    - 11.4 Ethidium Bromide 10 mg/ml (Sigma Aldrich Inc®, USA)
    - 11.5 Gel electrophoresis buffer (TAE)
    - 11.6 Loading dye (Fermentus®, Canada)
    - 11.7 *pfu* DNA polymerase and mixture (Fermentus®, Canada)
12. PCR cabinet (Biometra®, Germany)
13. PCR tubes and Microcentrifuge tube 1.5 ml
14. Vortex, Model K 550-GE (Scientific Inc., USA.)
15. Shaking Incubator, Model 311DS (Labnet International Inc., USA.)

APPENDIX B

Reagents and preparations

Reagents for agarose gel electrophoresis

1. 10 mg/ml Ethidium bromide

- Ethidium bromide	1	g
- Distilled deionized water	1,000	ml

Add 1 g of ethidium bromide to 100 ml of distilled deionized water. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap container in aluminum foil or transfer to a dark bottle and store at room temperature.

2. 50X TAE (Tris-Acetate buffer) 1000 ml contains

- Tris base	242.0	g
- Glacial acetic acid	57.1	ml
- 0.5 M EDTA pH 8.0	100.0	ml
- Distilled deionized water	1,000	ml

Add 242 g of Tris base, 57.1 ml of Glacial acetic acid and 100 ml of 0.5 M EDTA pH 8.0 to 500 ml of distilled deionized water and then adjusts the final volume to 1,000 ml. Sterilize the solution by autoclaving.

3. 0.5 M EDTA, pH 8.0 1000 ml contains

- Disodium ethylene diamine tetraacetate. $2H_2O$	186.1	g
- Distilled deionized water	800.0	ml

Add 186.1 g of disodium ethylene diamine tetraacetate. $2H_2O$ to 800 ml of distilled deionized water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to 8.0 by the addition of NaOH.

4. 1 M Tris HCl, pH 8.0 1000 ml contains

- Tris (ultrapure)	121.1	g
- Distilled deionized water	800.0	ml

Dissolve 121.1 g of Tris in 800 ml of distilled deionized water. Adjust the pH to 8.0 by adding conc. HCL 42.0 ml and then adjust the final volume to 1,000 ml. Sterilize the solution by autoclaving and store the solution at room temperature.

APPENDIX C

Locations of primers on the corresponding genes designed in this study

O-1C$_{124}$-F and O-1C$_{124}$-R (NK61) primers

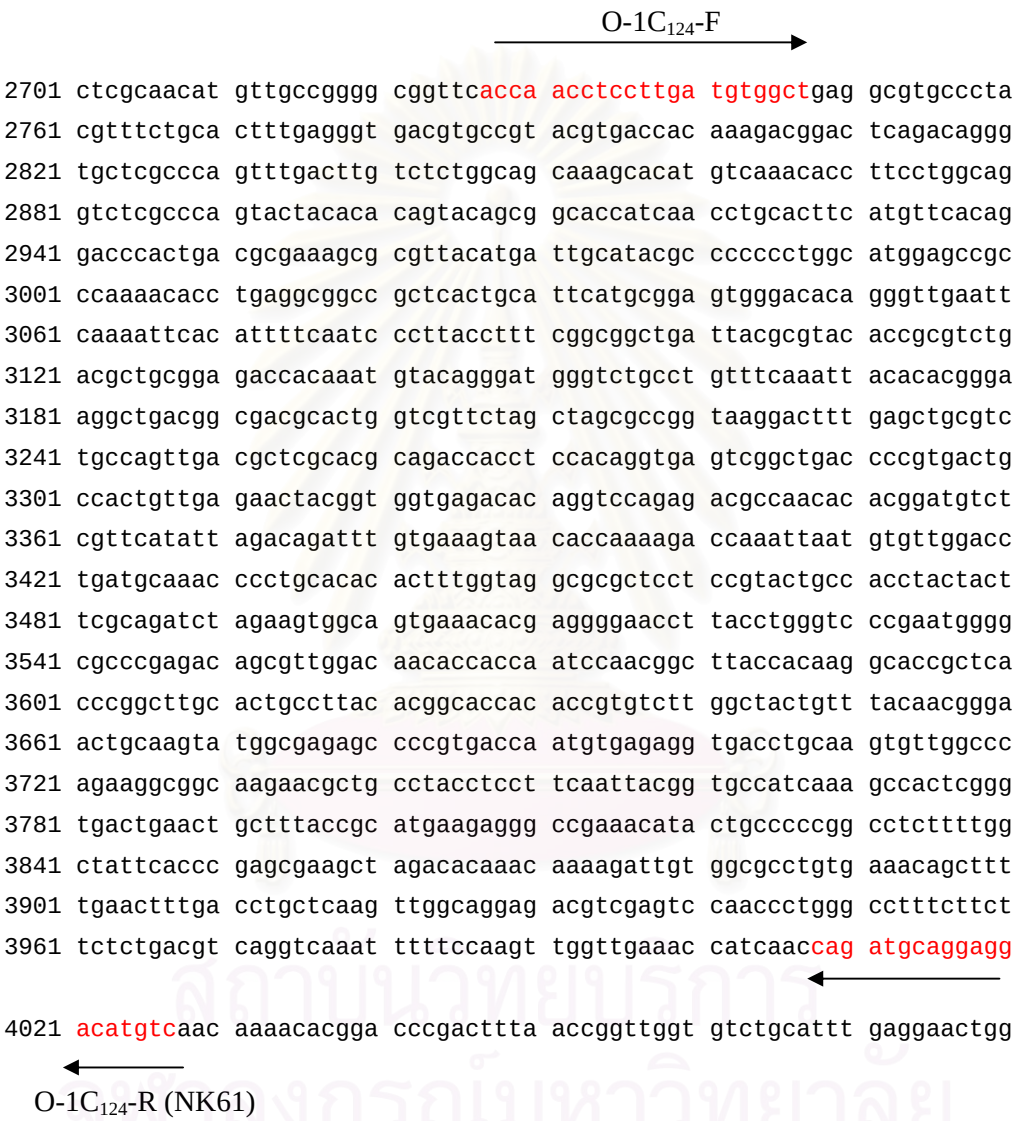


Figure A. Locations and nucleotide sequence of O-1C$_{124}$-F and O-1C$_{124}$-R (NK61) primers on VP3 – 2B gene of FMDV type O isolate Tibet /CHA/99 (Genbank accession number AJ539138). The arrows indicated position and direction of primers.

3ABC-F and 3ABC-R primers

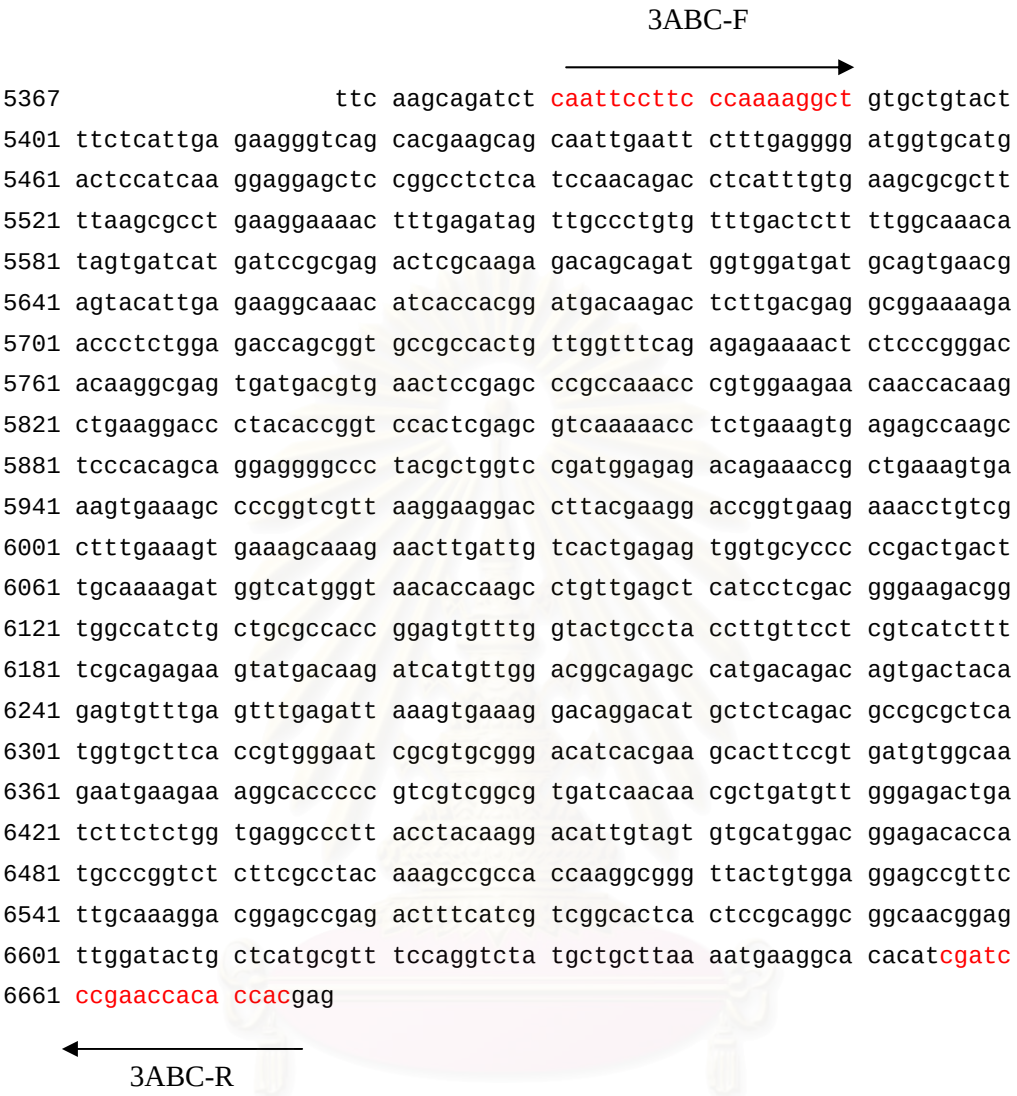
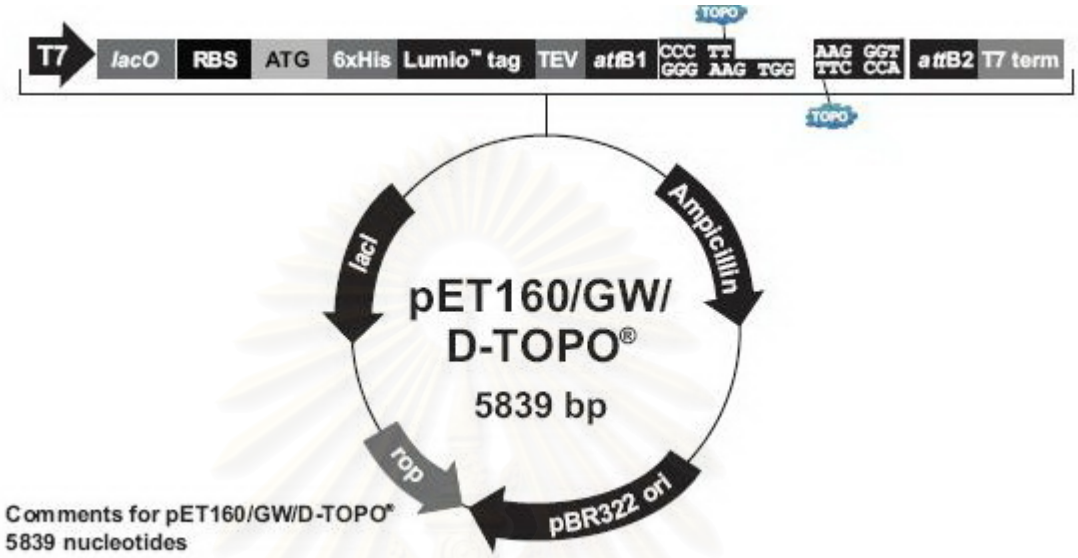


Figure B. Locations and nucleotide sequence of 3ABC-F and 3ABC-R primers on 3ABC gene of FMDV type O isolate Tibet /CHA/99 (Genbank accession number AJ539138). The arrows indicated position and direction of primers.

APPENDIX D

Physical map of plasmid pET160/GW/D-TOPO



Comments for pET160/GW/D-TOPO®
5839 nucleotides

T7 promoter/priming site: bases 21-40
lac operator (lacO): bases 40-64
Ribosome binding site (RBS): bases 94-101
Initiation ATG: bases 109-111
Polyhistidine (6xHis) region: bases 112-129
Lumio™ tag: bases 142-159
TEV recognition site: bases 169-189
*att*B1 site: bases 196-220
TOPO® recognition site1: bases 232-236
Overhang sequence (c): bases 237-240
TOPO® recognition site 2: bases 241-245
*att*B2 site: bases 257-281
T7 transcription termination region: bases 307-435
T7 reverse priming site: bases 346-365
bla promoter: bases 740-838
Ampicillin (*bla*) resistance gene: bases 839-1699
pBR322 origin: bases 1844-2517
ROP ORF (c): bases 2888-3079
lacI ORF (c): bases 4391-5482

(c) = complementary strand

BIOGRAPHY

Miss Kulisara Marupanthorn was born on September 1, 1981 in Ayutthaya, Thailand. She graduated from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2005. After that, she worked as a instructor at Rajamangala University of Technology Tawan-ok. She enrolled the degree of Master of Science in the Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University in 2006.