## ACKNOWLEDGMENTS

I am overwhelmed with gratitude for my thesis advisor, Assc. Prof. Dr. Worawidh Wajjwalku who has given me graduate study opportunity for his help, encouragement, careful guidance, countless technical helpful suggestion and his patience. I would sincerely like to thank my committee members Asst. Prof. Dr. Theerapol Sirinarumitr, Asst. Prof. Dr. Thavajchai Sakpuaram and Assc Prof. Dr. Saree Donkaewbua for their support throughout my graduate study.

I would also like to acknowledge faculty of Veterinary Medicine, Kasetsart University and Regional Reference Laboratory for FMD in South East Asia, Pakchong, Nakhon Ratchasima for the educational opportunity and the partial financial support.

I thank many people from Kamphaengsaen Veterinary Diagnostic Center in Kamphaengsaen campus for assistance during my study. I thank my colleagues who always support me.

Finally, my appreciation devotes to my parent and my family who always give me heartful supports.

Preeda Lertwatcharasarakul

May 2006

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ANCR | = | Amnat Charoen |
| BRR | = | Buri Ram |
| CM | = | Chiang Mai |
| CP | = | Chumphon |
| CR | = | Chiang Rai |
| CYP | = | Chaiyaphum |
| KCNBR | = | Kanchanaburi |
| KLS | = | Kalasin |
| LOEI | = | Loei |
| LP | = | Lampang |
| LPN | = | Lumpun |
| MDH | = | Mukdahan |
| MHS | = | Mae Hong Son |
| MRS | = | Maha Sarakham |
| NAN | = | Nan |
| NK | = | Nong Khai |
| NKPT | = | Nakhon Pathom |
| NKPN | = | Nakhon Phanom |
| NKSW | = | Nakhon Sawan |
| NRTW | = | Narathiwat |
| PC | = | Phichit |
| PCBR | = | Phetchaburi |
| PK | = | Phuket |
| PSNL | = | Phitsanulok |
| PTN | = | Pattani |
| PY | = | Phayao |
| RCBR | = | Ratchaburi |
| SK | = | Songkhla |

## LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| SKNK | = | Sakon Nakhon |
| SSK | = | Si Sa Ket |
| UB | = | Ubon Ratchathani |
| UDTN | = | Udon Thani |
| UTRD | = | Uttaradit |
| YL | = | Yala |
| YST | = | Yasothon |
| µl | = | microliter |
| ml | = | milliliter |
| µg | = | microgram |
| mg | = | milligram |
| bp | = | base pair |
| mM | = | milimolar |
| M | = | molar |
| DNA | = | Deoxyribonucleic acid |
| RNA | = | Ribonucleic acid |
| PCR | = | Polymerase Chain Reaction |
| EtBr | = | Ethidium Bromide |
| UV | = | ultraviolet |
| SDS-PAGE | = | Sodium Dodecyle Sulfate Polyacrylamide Gel Electrophoresis |
| rpm | = | round per minute |
| °C | = | Degree Celsius |
| *E.coli* | = | *Escherichai coli* |

# STUDY ON VP1 VARIATION OF FOOT-AND-MOUTH DISEASE VIRUS THAILAND'S ISOLATES AND EVALUATION OF SYNTHETIC PEPTIDE VACCINE FOR FMDV SEROTYPE O AND THE DEVELOPMENT OF THE 3AB PROTEIN SERODIAGNOSTIC ASSAYS

## INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of livestock animals that has potential for very serious and rapid spreading. Foot-and-mouth disease virus (FMDV) is an RNA virus which has high mutation rate. The virus has been classified into seven serotypes and several subtypes. Convalescent animals can become carriers of this disease where virus can be replicated and mutated. Surveillance, restricted movement, slaughtering and prevention are the important strategies for disease controlling.

The FMDV relationship has been evaluated and classified by nucleotide sequence alignments which presents on phylogenetic tree. The VP1 gene contained hypervariation and major antigenic site is used to evaluation of genetic variation. The phylogenetic tree is required to explain the virus epidemiology.

Precise and rapid diagnosis of FMD lead to the decision of control strategies as the diagnosis method has been developed. Nonstructural proteins (NSPs) are used for investigation a viral replication, due to antibodies from infected animals are reacted with NSPs (2C, 3A, 3B, 3AB and 3ABC), but not in vaccinated animal. Therefore, nonstructural proteins have been used to discrimination antibodies of infected from vaccinated animals. The majority of techniques are presented in enzyme linked immunosorbent assay (ELISA). Moreover, the monoclonal antibody is also proposed for diagnosis as well as investigation of the epitope.

Inactivated vaccine is commonly used for the disease prevention. However, several types of vaccine have been developed to increase safety and efficiency. Synthetic peptide vaccine

is also developed and has been evaluated for the disease protection. Synthetic peptide vaccine based on G-H loop of VP1 gene is mainly chosen for peptide synthesis which has been proven to protect animals.

This report describes (1) investigation of genetic and antigenic variation of outbreak viruses during 1998 to 2005, (2) evaluation the efficacy of the synthetic peptide vaccine. (3) development of ELISA to detect virus replication and (4) producing monoclonal antibodies against 3AB protein for the agent detection. The knowledge from this study is important for the understanding of the virus epidemiology in Thailand, the development of the precise and rapid diagnostic techniques for the surveillance of infected and carrier animals and the evaluation of the efficacy of the synthetic peptide vaccine for FMD control in Thailand.

# OBJECTIVES

1. To investigate the genetic and antigenic variation of serotype O, A and Asia1 of FMDV during 1998 to 2005.

2. To evaluate the antibody response in swine after immunized with synthetic peptide vaccine.

3. To develop ELISA based on 3AB recombinant protein to discriminate of infected and vaccinated animals.

4. To produce MAb against 3AB recombinant protein to detect of virus by IPMA technique.

# LITERATURE REVIEW

## 1. Foot-and-Mouth Disease

Foot-and-mouth disease (FMD) is a high contagious viral disease and harmful to domestic cloven-hoofed animals worldwide. Susceptible animals are cattle, pig, goat, sheep, and etc., which are important livestock animals. FMD is very serious transmissible disease for socio-economic and public health. FMD has also an impact to the international trade in animals and animal products. It is classified as list A disease by Office International des Epizootic (OIE) that has potential for very serious and rapid spreading. The incubation period of disease is 2-14 days (Mann and Sellers, 1989; Davies, 2002; Alexandersen *et al*., 2003; Sutmoller *et al*., 2003).

Typical cases of FMD are characterized by vesicular formation of the feet, buccal mucosa and mammary glands. The virus destroys the epithelium around the mouth, on the tongue and on the cloven hoofed of infected animals, which could contaminate to animal products for instance meat, milk, sperm, ova and embryo. The disease causes the reduction in milk production, skin quality, abortion and breeding problems. The severity of disease varies according to the strain of viruses and susceptibility of infected animals. Cattle are usually the most severely affected species while goat and sheep can be infected with mild or absent clinical signs. The disease is low mortality, but the high mortality caused by myocarditis is often found in young animals, and high morbidity typically approaches 100 % in unprotected herds (Mann and Sellers, 1989; Davies, 2002; Alexandersen *et al*., 2003).

FMD is naturally transmitted by a variety of routes such as inhalation, ingestion and entry via damaged epithelium. The virus can be found in secretions of infected animals before an occurrence of clinical signs (Mann and Sellers, 1989; Davies, 2002; Alexandersen *et al*., 2003; Sutmoller *et al*., 2003). The viral transmission via airborne could also be detected within 10 kms from the outbreak area (Mann and Sellers, 1989).

Convalescent animals may become carriers for a long time. About 50% of infected cattle may remain as a carrier stage for 9 months and sometimes extend to 2 years or more (Mann and Sellers, 1989; Prato Murphy *et al*., 1994). The virus is recovered from the esophageal pharyngeal areas of persistent cattle up to 539 days postinfection (Gebauer *et al*., 1988). For 50% of sheep and goats, the carrier period is 9 weeks and in some cases may extend to 9-11 months (Mann and Sellers, 1989). The carrier period appears at least 5 years in African Buffalo (Davies, 2002). Persistent infection of animal with FMDV is not only an important natural reservoir of the virus but also a potential source of antigenically variant virus, which is one of the main problems to design an effective vaccine (Gebauer *et al*., 1988; Dawe *et al*., 1994; Bastos *et al*., 1999; Golde *et al.*, 2005). Viruses from the carrier cattle show point mutation in nucleotide sequences that represents the mutation rate of 0.9 $x10^{-2}$ to 7.4 $x10^{-2}$ substitutions per nucleotide per year, and 59% of the nucleotide change leads to amino acid substitutions (Sobrino *et al*., 1986; Gebauer *et al.*, 1988).

Surveillance, restricted movement, slaughtering and vaccination are important strategies for controlling (Golde *et al.*, 2005). The inactivated vaccine is commonly used, but vaccine production produced from live virus may cause contamination of virus for environment during production and virus outbreak, because there may be incomplete inactivated vaccine viruses. Many kinds of vaccine have been developed for disease protection in laboratory animals and susceptible animals such as synthetic peptide vaccine (Bittle *et al*., 1982; Van Lierop *et al*., 1992, 1994; Volpina *et al.*, 1996, 1999; Wang *et al*., 2001, 2002; Rodriquez *et al.*, 2003), DNA vaccine (Wong *et al*., 2000, 2002; Zhang *et al*., 2003), recombinant viral VP1 from transformed *E.coli* (Bayry *et al.*, 1999; Wang *et al*., 2003) and recombinant viral VP1 from plants (Usha *et al.*, 1993; Carrillo *et al*., 1998; Wigdorovitz *et al*., 1999; Dus Santos *et al.*, 2001).

## 2. Foot-and-Mouth Disease Virus

The causative agent of the disease is foot-and-mouth disease virus (FMDV) which is the member of Aphthovirus of *Picornaviridae*. It is sensitive and rapidly inactivated at elevated temperatures. The optimal pH of virus is 7.2-7.6 (Mann and Sellers, 1989; Domingo *et al*., 2002; Sutmoller *et al*., 2003). The virus replication occurs in the cytoplasm of infected cells (Polatnick and Wool, 1982; Porter, 1993) which RNA synthesis becomes significant at 2.5 hours postinfection and peaks at 3.5 hours (Polatnick and Wool, 1982). Extensive serological and antigenic diversity lead the occurrence of seven serotypes, namely, O, A, Asia1, C, SAT1, SAT2 and SAT3 (SAT = South African Territories), and more than 60 subserotypes have been reported around the world. Three outbreak serotypes in Thailand are recognized as O, A and Asia1. FMD has been endemic in Thailand for more than 50 years. Type A, Asia1 and O were initially reported in 1953, 1954 and 1957, respectively (Chaisrisongkram, 1993). The serotype O and A have been reported as the most common, but serotype Asia 1 has not been reported since 1999 (Linchongsubongkoch *et al*., 2003).

### 2.1 Genome of FMDV

The FMDV is non-enveloped particle of icosahedral symmetry and its genome is a single positive stranded RNA (ssRNA) about 8,500 nucleotides in size (Sobrino *et al.*, 1986; Domingo *et al*., 2002, 2003; Mason *et al.*, 2003). The ssRNA carries a poly A tract at its 3' end and viral genome protein (VPg) at the 5' end. The VPg is required for viral replication. FMDV also contains an internal poly C tract within the 5' untranstranslated region (5'UTR). The virus translates to a polyprotein, and is cleaved into four primary cleavage products; the amino terminal L, P1-2A, 2BC and P3. Subsequently, the proteins are cleaved into two main functional proteins; structural protein (SP) and nonstructural protein (NSP) (Figure 1) (Forss *et al*., 1984; Robertson *et al*., 1985; Grubman *et al.*, 1987; Porter, 1993; Saiz *et al*., 2001; Domingo *et al*., 2002; Clavijo *et al*., 2004b; Carrillo *et al.*, 2005).

### 2.1.1 Non Structural Proteins (NSPs)

The nonstructural proteins conserve across all serotypes, are involved in viral replication and inhibition of host cell function (Grubman *et al.*, 1987; Porter, 1993; Li *et al.*, 2001; Capozzo *et al.*, 2002; Domigo *et al*., 2002; Pacheco *et al.*, 2003; Clavijo *et al.*, 2004b). FMDV genome contains three proteases (Leader (L), 2A and 3C). The L gene is located at the 5' end of the open reading frame. It contains two initiation codons. This protease initiates cleavage by separating itself away from P1 at the L/P1 junction. In addition, the protease cleaves eukaryotic initiation factor 4G (eIF-4G) during virus replication (Strebel and Beck, 1986; Devaney *et al.*, 1988; Saiz *et al.,* 2002; Clavijo *et al.*, 2004b). The 3C cleaves between 2BC and P3 from polyprotein, and is responsible for the cleavage of P1 into AB (VP0), 1C (VP3), and 1D (VP1). The 1A/1B (VP4/VP2) cleavage occurs at the late stage in virus morphogenesis, and is concomitant with encapsidation of virion RNA. Furthermore, the 3C protease cleaves histone 3 (H3) protein of host-cell and may be involved in the shutdown of host cell transcription (Capozzo *et al*., 2002; Clavijo *et al.*, 2004b). The cleavage between the 2A/2B junction is mediated by the 2A polypeptide separating itself (Donnelly *et al.,* 2001; Clavijo *et al.*, 2004b).

The RNA polymerase (3D) and VPg (3B) directly involve in the synthesis of RNA. The VPg can be uridylylated by RNA polymerase which can serve as a primer for RNA replication. The FMDV contains three tandem copies of 3B. The level of viral infectivity correlates with number of copies present in the RNA (Saiz *et al.,* 2002). The hydrophobic protein 3A and its precursor 3AB are associated with intracellular membranes in cultured cells, which inhibit protein secretion (Porter, 1993; Clavijo *et al.*, 2004b). The 3A may induce membrane permeability by pore forming in infected cell and also reduces the presentation of new antigens by preventing the expression of major histocompatibility complex (MHC) class I molecules on surface of infected cell (Clavijo *et al*., 2004b). In Taiwan in 1997, the type O virus, which infected pigs but not cattle, had an altered 3A protein. Its codons 93 to 102 deletion previously is found in O/YUN/TAW/97 (Beard and Mason, 2000; Knowles *et al.*, 2001; Pacheco *et al.,* 2003; Feng *et al.,* 2004)). However, full-length 3A coding region and nt 133-143 deletion from Vietnamese and Cambodian isolates are well replicated in kearatinocytes obtained from both pigs

and cattle (Knowles *et al*., 2001; Pacheco *et al.*, 2003). The NSP encoded by the 2BC region may play a role in increasing membrane permeability by disrupting the intracellular $Ca^{2+}$ homeostasis. Moreover, the protein 2C implicates in virus encapsidation and the formation of viral replication vesicle (Vance *et al.*, 1997; Clavijo *et al.*, 2004b).

### 2.1.2 Structural Proteins (SPs)

Viral structural proteins consist of capsid protein, VP1, VP2, VP3 and VP4, which are concerned about structural proteins, epitopes and receptors. The FMDV is 30 nm in diameter and consists of 60 copies of each SP. VP1, VP2 and VP3 are surface oriented whereas VP4 is internal (Saiz *et al.,* 2002). Capsid proteins are contained many T and B-cell epitopes. These epitopes can induce antibodies against FMDV. The VP1 have been exhibited a major antigenic site and high variation regions (Bittle *et al*., 1982; Robertson *et al*., 1983; Meloen and Barteling, 1986; Thomas *et al*., 1988a; Acharya *et al*., 1989; Collen *et al*., 1991; Martinez *et al*., 1991; Van Lierop *et al*., 1992, 1994; Mateu *et al.*, 1994; Rieder *et al.*, 1994; Dunn *et al.*, 1998; Sanz-Parra *et al.*, 1998; Verdaquer *et al*., 1998; Blanco *et al.*, 2000; Filgueira *et al.*, 2000; Marquardt *et al*., 2000; Marquardt and Freiberg, 2000).

Four antigenic sites are defined on type O FMDV. Two of them are located on VP1, the third site is on VP2, and the last one is on VP3 (Kitson *et al.*, 1990). Critical amino acid residues (144,147,148, 154 and 208) of antigen site one are located in the well known regions 133–160, currently called G-H loop, and 200- 213 of VP1. The homology in c-terminal with respect to nucleic acid sequence is less than 60% among serotype (Bittle *et al*., 1982; Duchesne *et al.*, 1984; Meloen and Barteling, 1986; Thomas *et al*., 1988a; Fox *et al*., 1989; Collen *et al*., 1991; Piatti *et al*., 1991; Rieder *et al.,* 1994; Zamorano *et al*., 1995; Suryanarayana *et al.,* 1999; Volpina *et al*., 1999; Wong *et al.*, 2000; Wang *et al*., 2003). The G-H loop also contains conserved sequence, Arg-Gly-Asp or RGD, a motif is a recognition element, receptor, in many integrin dependent cell adhesion processes (Acharya *et al.*, 1989; Fox *et al*., 1989; Logan *et al*., 1993; Verdaquer *et al.*, 1995; McKenna *et al*., 1996; Rieder *et al.*, 1996; Martinez *et al.*, 1997; Leippert and Pfaff, 2001). The synthetic peptides containing RGD sequence and antibody against the

RGD region of VP1 inhibit attachment of FMDV to baby hamster kidney (BHK) (Fox *et al*., 1989). Experimentally, the virus deleted RGD can not infect in culture cell, mouse and swine (McKenna *et al*., 1996). In the region 43 – 60 of VP1, amino acid residues 43, 44, 45 and 48 are involved in antigenic site 3. The fifth neutralizable site is identified on residue 149 in antigenic site 1 of VP1 and represents a conformational epitope (Crowther *et al.*, 1993). The majority of vaccine production is based on VP1 protein (Bittle *et al*., 1982; Usha *et al*., 1993; Van Lierop *et al.*, 1994; Volpina *et al.*, 1996, 1999; Carrillo *et al*., 1998; Wigdorovitz *et al*., 1999; Wong *et al*., 2000, 2002; Dus Santos *et al.*, 2001; Wang *et al*., 2001, 2002; Rodriquez *et al.*, 2003; Zhang *et al*., 2003).

The major antigenic site of serotype A also locates in the G-H loop of VP1. The critical positions contain residues 83, 139,140, 141, 149 153 and 173 which are identified by neutralization escape mutants (Baxt *et al.*, 1984, 1989; Thomas *et al*., 1988b; Tosh *et al.*, 2002). Monoclonal antibodies (MAbs) are investigated of antigenic sites in serotype A10 that are divided into two major antigenic sites. The first site is trypsin sensitive which includes amino acid residues 140-160 of VP1. The second site is trypsin insensitive which is found in VP3. Two minor sites are located near residue 169 of VP1 and on the C terminus of VP1 (Thomas *et al*., 1988b). Additionally, the nucleotide sequencing (serotype A12) is placed at least three epitopes of the major epitope site on VP1 which is estimated by neutralizing monoclonal antibodies (Baxt *et al.,* 1989).

In serotype Asia1, Mabs recognizing trypsin sensitive epitope usually bind the residues 146 (methionine) and 153 (leucine) of VP1, but not react both residues isolated from Thailand which are substituted by leucine and valine (Gurumurthy *et al*., 2002).

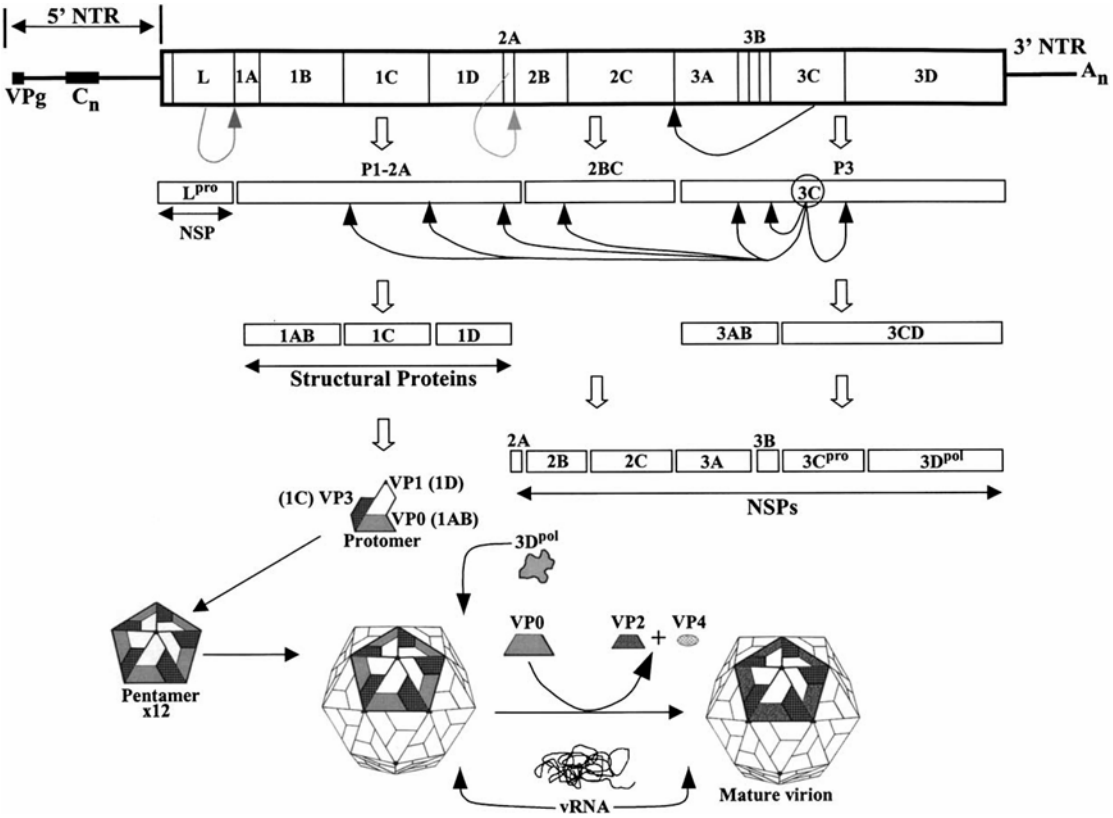



Figure 1 Genomic organization of FMDV. The 5' non-translated region (NTR) is capped by small protein VPg (3B) and contains a poly I tract. Polyprotein organize are shown boxed areas. Arrows indicted sites of cleavage. P1-2A is the precursor of the structural proteins. 2BC and P3 are the precursor of the non structural protein. After polyprotein are completely processed by viral protease, each structural protein is assembled to protomer. Five protomers assemble into pentamer, and 12 pentamers assemble subsequently with viral RNA into mature virus particle.

Source: Clavijo (2004b)

## 2.2 Molecular Epidemiology

Virus evolution and molecular epidemiology are a theoretical background for investigation of evolutionary pathways, genetic variability and predicting the relationship for virus study reviewed by Vandamme, (2002). There are crucial to design for better public health and veterinary medicine practices. Viruses do not leave fossils then the only way to track down a virus history is through its imprint on the viral genome. Phylogenetic analysis allows mapping by constructing trees, predicting the relationship. The branches that fall together can represent particular viral subtypes (Stram *et al*., 1995; Freiberg *et al*., 1999; Tsai *et al*., 2000; Vandamme, 2002). The fifteen percentages of nucleotide divergence between isolated viruses have been used as cut off value to define the genotype difference. Five percentages or less in nucleotide sequences belong to closely related viruses (Tsai *et al.*, 2000; Samuel and Knowles, 2001; Gurumurthy *et al*., 2002). The FMDV evolution is strongly influenced by high mutation and a quasispecies dynamics (Domingo *et al*., 2003). The synonymous mutations in FMDV play a major role in evolution of the viruses which are generated during replication (Saiz *et al*., 1993). The greatest variation in the genome is occurred in the region coding for the structural proteins (Domingo *et al.*, 2003).

### 2.2.1 Serotype O

The O serotype of FMDVs is divided into 8 genotype based on 469-639 of VP1 nucleotide sequence when a value of 15% nucleotide divergence is proposed. It is consisted of Europe–South America (Euro-SA) topotype, Cathay topotype, West Africa (WA) topotype, East Africa (EA) topotype, Middle East–South Asian (ME-SA) topotype, South-East Asia (SEA) topotype, Two Indonesian topotype (ISA-1 and ISA-2) (Figure 2) (Samuel and Knowles, 2001; Knowles and Samuel, 2003).

The Euro-SA topotype includes viruses which occur in Europe. Majority of viruses in Cathay topotype are reported in Philippine, Hong Kong and China. The WA topotype is originated from Ghana, Ivory Coast and Guinea. The EA topotype is represented by viruses isolated in Kenya and Uganda. The ME-SA viruses are originated from Indian subcontinent and

the Middle East Asia including the dominant outbreak of a pandemic strain in Taiwan and Japan during 1999 and 2000 is currently named Pan Asia (Tsai *et al.,* 2000; Kweon *et al.,* 2002; Mason *et al.*, 2003). The Pan Asia O strain is first identified in Northern India before spreading westward to Bulgaria and Greece in 1996 (Knowles *et al.*, 2005). The viruses of SEA topotype are seen in South-East Asia for instance Myanmar, Thailand, Cambodia, Malaysia, Loas and Vietnam. The ISA-1 and ISA-2 topotypes are comprised viruses isolated in Indonesia but different years (Samuel and Knowles, 2001).

### 2.2.2 Serotype A

Serotype A viruses are distributed into 10 major genotypes (designated as I-X) based on complete VP1 nucleotide sequences (Figure 3) (Tosh *et al.,* 2002.). Genotype I is consisted of 16 viruses isolated in India, and includes viruses from Columbia, Greece, India, Italy, Morocco, the Netherlands and Spain. Genotype II is contained strains from Argentina, Brazil, United Kingdom and Venezuela. Genotype III is isolated from China. Genotype IV spreads in a large area, Bangladesh, India, Iraq and Former USSR. The strains from Europe (Spain/73) and South America (Argentina/87) are genotype V. Genotype VI is isolated from India including one vaccine strain of India (IND490/97). Viruses from Albana (Albenia/96) and India are member of genotype VII. Genotype VIII are found in Iran (Iran/87), Saudi Arabia and Turkey. Viruses from Thailand and Malaysia are genotype IX. Finally, Genotype X is found in Iran (Iran/4/98 and Iran/17/97).

### 2.2.3 Serotype Asia1

The previous report presented the outbreak of three viruses isolated from India, Thailand and Israel is indicated the circulation of three genotypes of serotype Asia1. These viruses are formed by three reaction patterns with the MAbs (Marquardt *et al*., 2000). Then, genetic diversity of FMDV serotype Asia1 is studied based on complete nucleotide sequence of the VP1 which can be divided into four genotypes (genotypes I-IV). The virus isolated from Pakistan (PAK/1/54) is formed genotype I. The viruses from Indian isolation including the two

vaccine viruses (IND 63/72 and IND 491/97) are formed a genotype II. Viruses from Israel (Shamir/89) and China are formed genotype III and IV, respectively. The rate of evolution of FMDV Asia 1 virus is estimated and found to be 2.7 $X10^{-2}$ synonymous substitutions per nucleotide per year. The nonsynonymous substitution rate is estimated to 1.1 X $10^{-3}$ substitutions per nucleotide per year. Mohapatra *et al.,* (2004) groups of viruses into five major lineages (A to E) based on percent nucleotide divergence in P1 region. The branch A is represented by the prototype strain PAK 1/54. Branch B and C consist of viruses from India. Branch D is group of Israel (LEB/83). And viruses isolated from Thailand and Turkey are formed to branch E. Moreover, the phylogenetic tree based on VP1 gene sequence is distributed into 6 lineages, designated as I-VI (Figure 4). The FMDV Asia1 prototype strain (PAK1/54) is formed lineage I. Isolates from Thailand and Turkey (THAI 5/96 and TUR 7/00) are formed lineage II. Isolates from Lebanon (LEB 83) are formed lineageIII. An isolate from China is formed lineage IV. The 54 isolates from India are found into 2 lineages (lineage V and VI) (Sanyal *et al*., 2004). Additionally, 603 nucleotides of Leader gene (L gene) are used for sequence and phylogenetic analyses, which are congruent with respect to major branching pattern of VP1, based on trees (633 nt.) (Mohapatra *et al*., 2002).

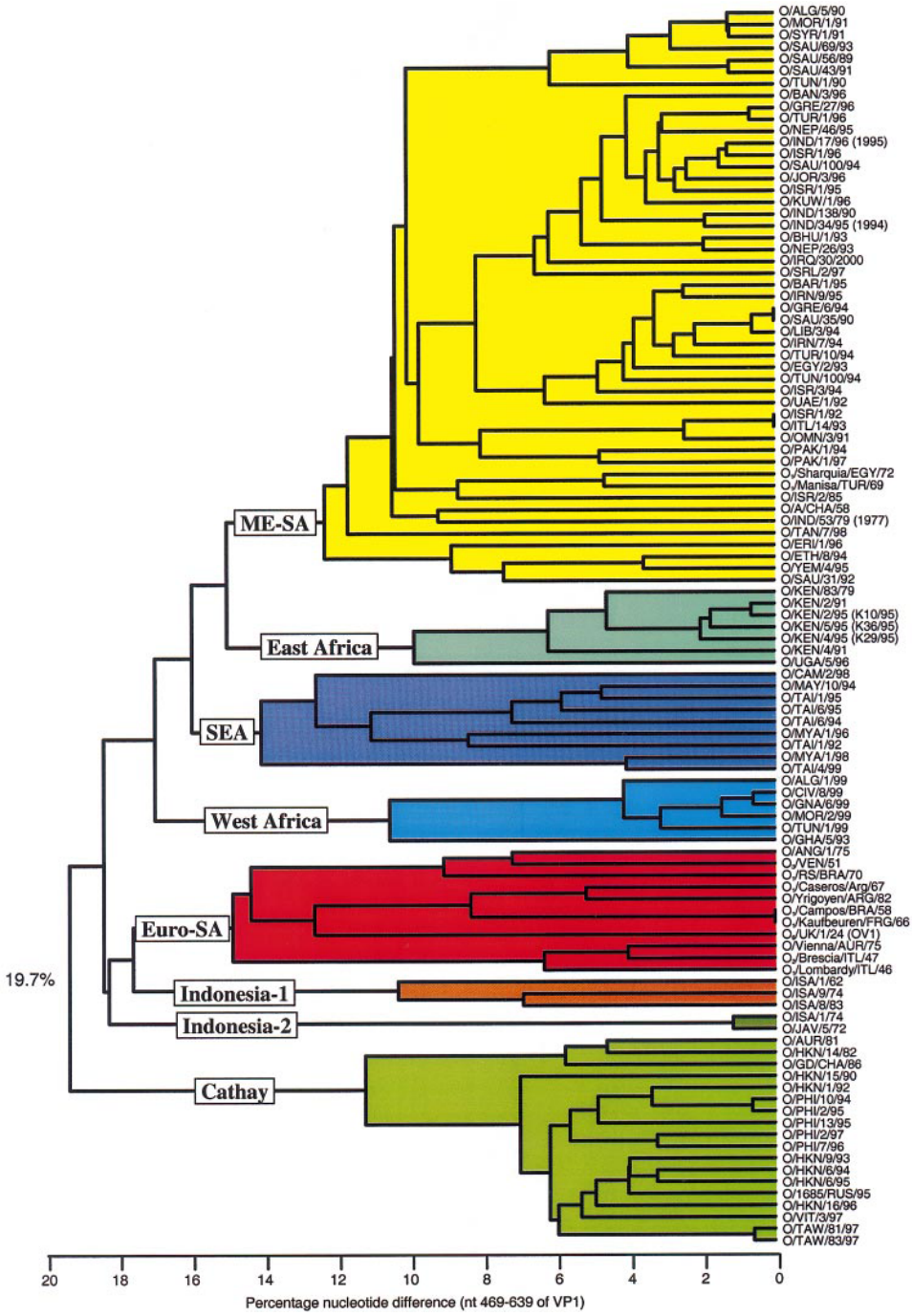



Figure 2 Phylogenetic tree of FMDV serotype O based on nucleotide sequence 469-639 of VP1. The viruses are divided into 8 topotypes.

Source: Samuel and Knowles (2001)

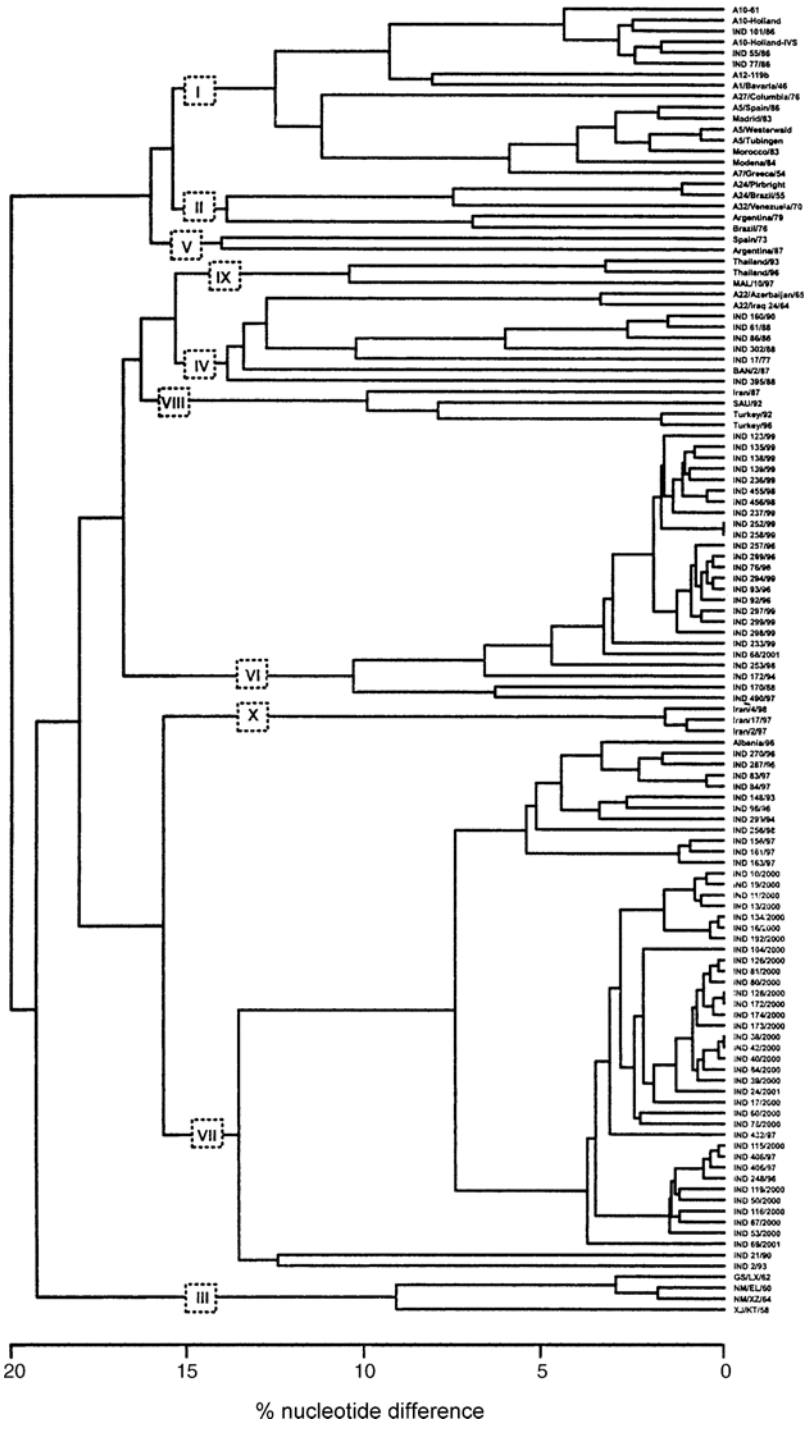



Figure 3 UPGMA tree constructed from VP1 gene sequence of serotype A isolates. Genotypes, designated as I-X are shown on the branches.

Source: Tosh (2002)

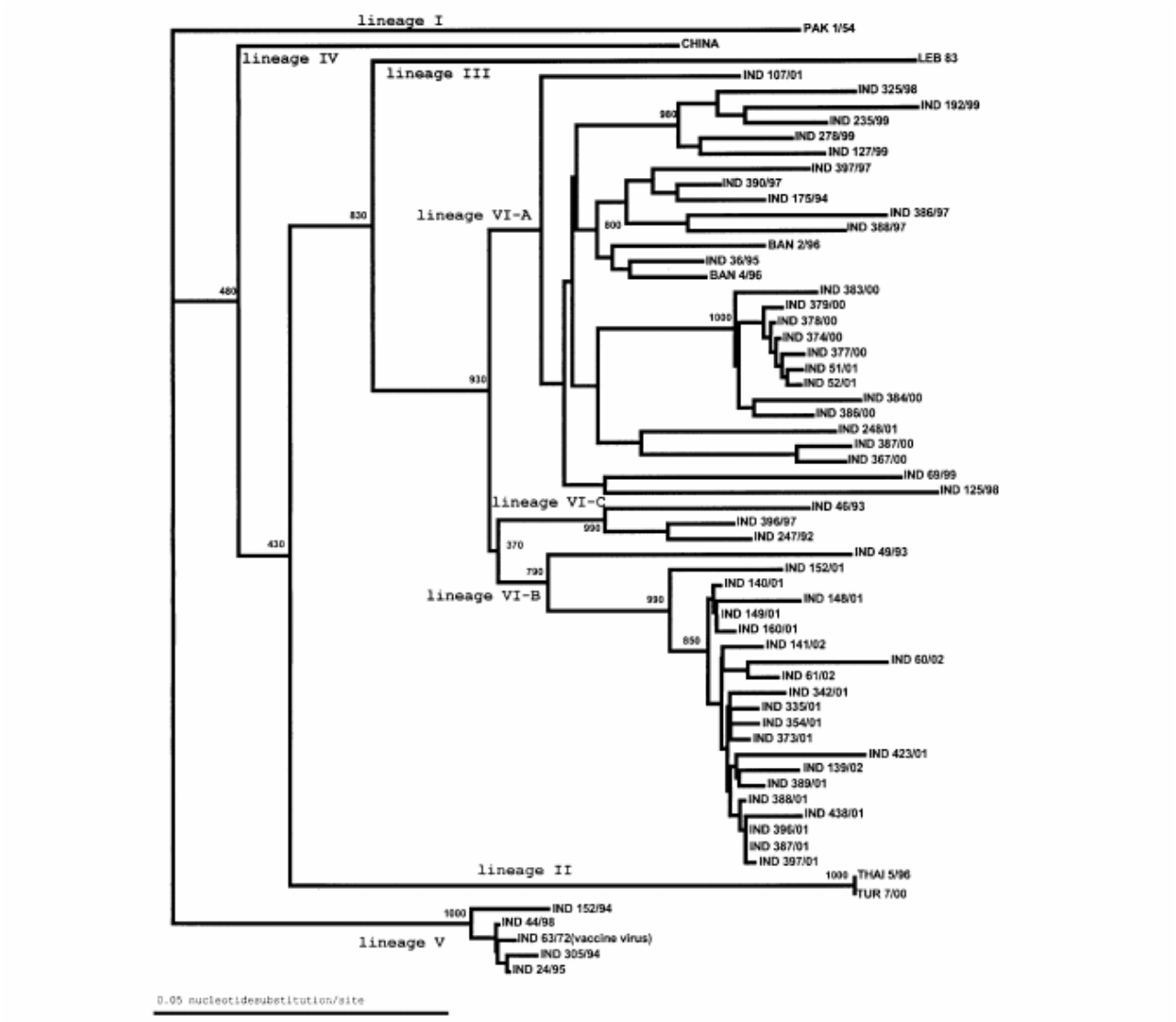



Figure 4 Neighbor-joining tree showing branching pattern of different Asia1 viruses in the 1D (VP1) region. The viruses are divided into 6 lineages (I-VI).

Source: Sanyal (2004)

### 2.3 The FMDV in Thailand

The Regional Reference Laboratory for FMD in South East Asia (RRL) located at Pakchong, Nakhon Ratchasima Province, Thailand is responsible for FMD diagnosis and serves as the national and regional laboratory. Viral relationship between field strain and vaccine strain has been estimated by viral neutralization (VN) and liquid phase blocking ELISA (LP-ELISA). Serological relationship (r-value) is the value indicated the virus relationship. The r-value is ranged into 3 levels which show the degree of similarity or close related virus. The r-value of Type O and Asia1 are found no antigenic change from vaccine strain (O/UDN/87 for serotype O and Asia1/PBR/85 for serotype Asia). Serotype Asia1 has not been reported since 1999. For serotype A, the r-value varies of antigenic from vaccine strain that has been changed seed virus according to r-value of outbreak strain, A/sakonakorn/97 in late 1997 and A/118/87 in 2002 (Linchongsubongkoch, 2003). Moreover, nucleotide sequences based on approximately 171 nucleotide (nt. 469-639) of VP1 are used for phylogenic analysis by using the Neighbor-joining method. The results show that FMDV serotype O of Thailand during 2001 – 2002 fall into South East Asia (SEA) topotype (Linchongsubongkoch, 2003; Thongtha, *et al.,*2003). The serotype A and Asia1 are found three and one groups, respectively (Linchongsubongkoch, 2003).

## 3. Laboratory Diagnosis

The diagnosis of FMDV can be divided by two main methods; the serological methods and the agent identification.

### 3.1 Serological methods

The serological methods are investigation of antibodies against FMDV such as Enzyme Linked Immunosorbent Assay (ELISA), Virus Neutralization (VN), Liquid-Phase Blocking Sandwich ELISA (Hamblin *et al*., 1986; Ferris and Dawson, 1988; Rodriguez *et al*., 1994; Bayry *et al*., 1999; Mackay *et al*., 2001; Chung *et al.*, 2002; Chenard *et al*., 2003; Clavijo *et al*., 2004a), Virus Infection-Associated Antigen (VIAA) test (Cowan and Graves, 1966; Pinto and

Hedger, 1978; Lowe and Brown, 1981; Neitzert, *et al*.,1991; Clavijo *et al*., 2004b) and Enzyme-Linked Immunoelectrotransfer Blot Assay (Bergmann *et al.*, 2000). These are available for epidemiological surveys and measurement of the vaccination's effectiveness.

Antibodies against nonstructural proteins (NSP) that covered 2C, 3B, 3AB, 3ABC and 3D (RNA polymerase or VIAA) can detect infected animals but vaccinated animals, because NSPs lost from vaccine procession (Rodriguez *et al*., 1994; Silberstein *et al.*, 1997; Sorensen *et al.*, 1998; Shen *et al*., 1999; Bergmann *et al*., 2000; Chung *et al.*, 2002; Hohlich *et al.*, 2003; Kweon *et al.*, 2003; Bronsvoort *et al*., 2004; Bruderer *et al*., 2004; Clavijo *et al*., 2004a; Lee *et al*., 2004; Moonen *et al*., 2004; Armstrong *et al*., 2005; Robiolo *et al.,* 2006). In present, NSPs have been used as vaccine marker that can identify infected animal sera with all seven serotypes because the NSPs are highly conserved among serotypes (Clavijo *et al*., 2004b). The NSPs have been produced by recombinant *E.coli* (Rodriguez *et al*., 1994; Silberstein *et al.*, 1997; Robiolo *et al.,* 2006), by recombinant baculoviruses in insect cell (Silberstein *et al.*, 1997; Sorensen *et al.*, 1998; Chung *et al.*, 2002; Kweon *et al.*, 2003) and by synthetic peptide (Shen *et al.*, 1999) for antibody detection. Additionally, the yield of soluble NSP 3D and 3AB in baculovirus expression system are about 10-20 times higher than of 3ABC, when measured by ELISA technique (Sorensen *et al.*, 1998). However, 3D can be detected in vaccinated animal (Silberstein *et al*., 1997; Sorensen *et al.*, 1998). Additionally, antibodies against 3AB and 3ABC proteins are produced by convalescent animals and carriers that can be detected from day 8 to more than 3 years after infection by ELISA techniques (Sorensen *et al.*, 1998; Chung *et al*., 2002).

### 3.2 Agent Identification

The second method is detection of causative agent. The brief details of available techniques are following. The viral isolation technique is done by virus intact propagation in permissive culture cells, for examples; baby hamster kidney (BHK)-21, foetal lamb lung (FLL) and primary calf thyroid cells. The virus involves cytopathic effect (CPE) in those cells after infection (Kongthon, 1993; Reid *et al*., 1999). Indirect sandwich test is enzyme linked immunosorbent assay technique using for the detection of FMDV (Roeder and Le Blanc Smith,

1987). The In situ hybridization technique is developed for investigation the presence of FMD virus RNA in tissue samples and infected cell (Zhang and Kitching, 2000). Nucleic acid sequence-based amplification (NASBA) technique is developed allowing the detection of foot and mouth disease virus genetic material in a range of sample material (Collins *et al*., 2002).

The Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) has been genetic material investigation of virus which is high sensitivity and accuracy. Universal primers and specific primers for each serotype are used for FMDV detection and typing (Amaral-Doel *et al*., 1993; Prato Murphy *et al*., 1995; Vangrysperre and De Clercq, 1996; Callens and De Clercq, 1997; Reid *et al*., 1998, 1999, 2000; Suryanarayana *et al*., 1999; Wajjwalku *et al*., 1999; Hofmann *et al*., 2000; Linchongsubongkoch *et al.*, 2000). This technique can be directly applied to vesicular fluid, tissues and esophageal-pharyngeal fluid of putative FMDV infected animals which can be investigated virus in carrier cattle more than 270 day post-infection (Amaral-Doel *et al.*, 1993; Prato Murphy *et al*., 1994; Reid *et al*., 1999). The specificity of reactions is applied for identification of other vesicular disease viruses, namely: swine vesicular disease virus and vesicular stomatitis virus (Reid *et al*., 2000). The primers are designed on locations of VP1, VP3, VP4 and 5' untranslated region (UTR) (Locher *et al.,* 1995; Reid *et al*., 1999; 2000). Validated RT-PCR procedures for the serotyping of FMD virus would best be used as a back-up diagnosis method to virus isolation and ELISA. However, serotyping by PCR is required nucleotide sequencing for confirmation. RT-PCR product has been used for cycle sequencing techniques to study genetic and antigenic variation and epidemiology of the virus (Stram *et al*., 1995; Pattnaik *et al*., 1998; Tsai *et al*., 2000; Samuel and Knowles, 2001).

The nucleotide sequences from direct sequencing of PCR products are shown by sequences alignment and phylogenetic grouping based on VP1 sequences which correlate well with classical serological and subtype classification (Saiz *et al*., 1992, 1993; Stram *et al*., 1995; Marquardt and Haas, 1998; Linchongsubongkoch *et al.*, 2000; Tsai *et al*., 2000; Huang *et al*., 2001; Knowles and Samuel, 2003; Mason *et al.*, 2003; Sanyal *et al*., 2004). Each serotype of virus is reported approximately 60% difference in VP1 nucleotide sequences (Cheung *et al*., 1983). Difference degree of VP1 nucleotide sequence at the mean 15% is indicated the specified

genotypes but 5% different nucleotide sequence indicates the close relationship among genotypic group (Marquardt and Haas, 1998; Samuel and Knowles, 2001). The identification of the genetic variation and its antigenic characterization are available for appropriate seed virus that are served as the tool for vaccine application and epidemiology (Beck and Strohmaier, 1987; Gebaues *et al*., 1988; Saiz *et al.,*1993; Stram *et al.,* 1995; Tsai *et al.*, 2000). The nucleotide sequencing data is useful for tracing the origins and extent of genetically distinct viruses causing outbreak. Full length of VP1 (639 nucleotides) (Tsai *et al*., 2000), 3'end of the VP1 gene (165 nt.) (Mohapatra *et al*., 2002), 181 bases from nt. 432 to 612, nucleotide 321 to 612 (Stram *et al.,* 1995) and Leader gene or L gene (Mohapatra *et al*., 2002; Tosh *et al.,* 2004) are used for nucleotide alignment.

Study in molecular epidemiology of FMDV outbreak in Thailand has been fairly reported, hence it is important for disease control, including selection of seed virus that closely related to outbreak strains. The goal of this study is aimed to described the molecular epidemiological investigation variation of nucleotide and amino acid sequences of serotype O, A and Asia1 during 1998-2005 based on sequence variation of G-H loop of VP1 gene of FMD viruses from Thailand epizootic. The results show that spread of virus by dendrogram and mutation of amino acid residues among viruses in major antigenic site of FMDV. Moreover, VP1 synthesis peptide-based vaccine including G-H loop of VP1 coupled with T-Helper epitope is studied the antibody response in swine after immunization. Diagnosis techniques are developed for detection of agent and detection of antibodies against FMDV. The recombinant 3AB protein expressed in *Escherichia coli* (*E.coli*) is improved for investigation of antibodies by ELISA technique to discriminate vaccine animals from infected normally animals. The recombinant 3AB protein is also used for immunized mouse for 3AB monoclonal antibodies production, which can be investigated FMDV in infected cell.

# MATERIALS AND METHODS

## 1. Phylogenetic Tree

### 1.1 Viruses

Viruses were isolated from different outbreak fields during 1998 to 2005 by Region Reference Laboratory for FMD in South East Asia, Pakchong, Nakhon Ratchasima and Kamphaengsaen Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen, Nakhon Pathom. The numbers of isolated viruses were showed in table 1. The cDNAs and PCR products were represented the viruses isolated in Region Reference Laboratory for FMD in South East Asia.

### 1.2 Virus Isolation

Baby Hamster Kidney (BHK) 21 was used for viral isolation of infected tissues at Kamphaengsaen Veterinary Diagnostic Center. Briefly, infected tissues were ground and centrifuged at 1500 rpms for 5 minutes. The supernatant of filtrates were filtered by 0.2 micron filter then 100 µl was added in the monolayer cells and incubated for 1 hour at 37°C, 5% $CO_2$. Dulbecco's Modified Eagle Medium (DMEM) containing 0.5% Fetal Bovine Serum (FBS) was added in all wells and incubated at 37°C in 5% $CO_2$. Cytopathic effect (CPE) was observed after 3-4 days of inoculation. If it was not found, supernatant was used for second passage in fresh monolayer cell and observed CPE after incubated for 3-4 days. Negative result displayed that CPE was not performed in second passage.

Table 1 Numbers of viruses isolated outbreak fields. The viruses were isolated from Region Reference Laboratory for FMD in South East Asia, Pakchong, Nakhon Ratchasima and Kamphaengsaen Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen, Nakhon Pathom.

| Serotype | Region Reference Laboratory for FMD in South East Asia (Number of samples) | Kamphaengsaen Veterinary Diagnostic Center (Number of Samples) | Total |
|---|---|---|---|
| O | 52 | 12 | 64 |
| A | 39 | 10 | 49 |
| Asia1 | 2 | 0 | 2 |
| | 93 | 22 | 115 |

### 1.3 RNA Extraction

The viral RNA was extracted from clinical tissue samples or infected cell culture using acid guanidine thiocyanate phenol chloroform RNA extraction method (Siebert and Chenchik, 1993). It was used as template for amplification by RT-PCR. Briefly, 500 µl of D-solution (Appendix A) was added to 100 µl of supernatant of infected cell (80% CPE) or tissue suspension and incubated for 5 minutes. Subsequently, the 50 µl of 2M sodium acetate (pH4.1), 200 µl of phenol and 200 µl of chloroform were added to the solution. It was centrifuged at 13,000 rpm for 10 minutes and aspirated the upper part to a fresh microtube. The RNA was precipitated by equal volume of cool absolute isopropanol and washed by 500 µl of 70% ethanol. After centrifuge, the RNA was dried at room temperature and resuspended in 50 µl cocktail of RobusT RT-PCR KIT (FINNZYMES®) (Appendix A).

### 1.4 Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

#### 1.4.1 Reverse Transcription

cDNA was synthesized by RobusT RT-PCR KIT (FINNZYMES®) (Appendix A) using hexamer primer in an extension for 30 minutes at 42°C, a denaturation for 5 minutes at 95°C and stored at 4°C.

#### 1.4.2 Polymerase Chain Reaction (PCR)

The cDNA was used as a template for PCR technique. It was amplified by Dynazyme EXT kit (FINNZYMES®) (Appendix A) using universal and specific primers. The nucleotide sequences of primers were shown in table 2. The reaction was performed in the DNA thermal cycle (model 2400; Perkin-Elmer, Norwalk, Conn.). For PCR amplification, 1 µl of cDNA was added to 9 µl of PCR cocktail (Appendix A). PCR condition was initiated by denaturation at 94°C for 5 minutes, and followed by 35 cycles of template denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55 °C and primer extension for 30 seconds at 72°C.

A final extension was carried out for 7 min at 72°C then stored 4°C. For detection, the PCR product was electrophorated in 1% agarose gel and stained with ethidium bromide (EtBr) (Appendix B). The DNA band was visualized by UV illumination.

### 1.5 Nucleotide Sequencing and Sequence Analysis

PCR products were labeled by ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystem) which were amplified by NK61 primer. Nucleotide sequencing was served at Bioservice unit, National Science and Technology Development. The nucleotide sequences of virus isolated from Thailand (Table 3 and 4) were aligned and analyzed with nucleotide sequences obtained from GenBank (Table 5, 6 and 7) by using Laser Gene biocomputing software package (DNASTAR) and MEGA3.1 program.

Table 2 Nucleotide sequences, location and serotype specific of oligonucleotide primers for RT-PCR and nucleotide sequencing

| Primer designation | Primer sequence (5'→3') | Serotype specific | Location | Approximated size (bp) |
|---|---|---|---|---|
| Positive sense primers | | | | |
| O-1C$_{124}$(ROD1) | TGTTGAAAACTACGGTGGTGA | 0 | VP1 | 700 |
| A-1C-612 | TAGCGCCGGCAAAGACTTTGA | A | VP3 | 800 |
| As1-1C$_{505}$ | TACACTGCTTCTGACGTGGC | Asia1 | VP3 | 900 |
| Negative sense primer | | | | |
| NK61[a] | GACATGTCCTCCTGCATCTG | Universal | 2B | - |

a = used for nucleotide sequencing

Source: Linchongsubongkoch (2000)

Table 3 History of serotype O isolated from Thailand during 1998 to 2005 at Region Reference Laboratory for FMD in South East Asia, Pakchong, Nakhon Ratchasima and Kamphaengsaen Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen, Nakhon Pathom.

| Virus Designation | Geographical origin | Species | Topotype |
|---|---|---|---|
| O/11/SK/98 | Songkhla | Cattle | SEA |
| O/12/SK/98 | Songkhla | Cattle | SEA |
| O/31/NKPT/00 | Nakhon Pathom | Cattle | SEA |
| O/28/PCBR/00 | Phetchaburi | Cattle | SEA |
| O/35/PCBR/00 | Phetchaburi | Cattle | SEA |
| O/F41/32/98 | Nakhon Pathom | Swine | SEA |
| O/04/PK/99 | Phuket | Cattle | SEA |
| O/02/MHS/99 | Mae Hong Son | Cattle | SEA |
| O/19/CR/00 | Chiang Rai | Cattle | SEA |
| O/42/NAN/00 | Nan | Buffalo | SEA |
| O/06/UTRD/00 | Uttaradit | Cattle | SEA |
| O/F45/17/02 | Ratchaburi | Swine | SEA |
| O/F45/14/02 | Nakhon Pathom | Swine | SEA |
| O/F45/16/02 | Ratchaburi | Swine | SEA |
| O/47/CP/01 | Chumphon | Cattle | SEA |
| O/54/CM/01 | Chiang Mai | Cattle | SEA |
| O/F48/04/05 | NK | NK | SEA |
| O/40/RCBR/00 | Ratchaburi | Cattle | SEA |
| O/10/LOEI/98 | Loei | Buffalo | SEA |
| O/26/YL/00 | Yala | Cattle | SEA |
| O/33/PTN/00 | Pattani | Cattle | SEA |
| O/34/YL/00 | Yala | Cattle | SEA |
| O/49/NRTW/00 | Narathiwat | Cattle | SEA |
| O/23/SK/00 | Songkhla | Cattle | SEA |
| O/30/SK/00 | Songkhla | Cattle | SEA |
| O/25/PTN/00 | Pattani | Cattle | SEA |
| O/13/PC/98 | Phichit | Cattle | SEA |
| O/45/KLS/00 | Kalasin | Cattle | SEA |
| O/46/CYP/01 | Chaiyaphum | Cattle | SEA |
| O/48/ANCR/01 | Amnat Charoen | Cattle | SEA |
| O/F44/03/01 | Ratchaburi | Swine | SEA |
| O/F44/04/01 | NK | Swine | SEA |

Table 3 (cont'd)

| Virus Designation | Geographical origin | Species | Topotype |
|---|---|---|---|
| O/F44/12/01 | Nakhon Pathom | Swine | SEA |
| O/F44/15/01 | NK | Swine | SEA |
| O/53/CR/01 | Chiang Rai | Swine | SEA |
| O/F44/02/01 | Nakhon Sawan | Swine | SEA |
| O/16/ANCR/00 | Amnat Charoen | Cattle | SEA |
| O/44/BRR/99 | Buriram | Cattle | SEA |
| O/37/CM/00 | Chiang Mai | Cattle | SEA |
| O/38/CM/00 | Chiang Mai | Cattle | SEA |
| O/15/SKNK/00 | Sakon Nakhon | Cattle | Pan Asia |
| O/01/CR/99 | Chiang Rai | Cattle | Pan Asia |
| O/09/CR/99 | Chiang Rai | Cattle | Pan Asia |
| O/43/PSNL/00 | Phitsanulok | Cattle | Pan Asia |
| O/08/CR/99 | Chiang Rai | Cattle | Pan Asia |
| O/20/PY/00 | Phayao | Cattle | Pan Asia |
| O/21/LP/00 | Lampang | Cattle | Pan Asia |
| O/14/NK/00 | Nong Khai | Cattle | Pan Asia |
| O/27/UD/00 | UdonThani | Cattle | Pan Asia |
| O/03/PY/00 | Phayoa | Cattle | Pan Asia |
| O/07/CR/99 | Chiang Rai | Cattle | Pan Asia |
| O/5/YST/99 | Yasothon | Cattle | Pan Asia |
| O/36/NK/00 | Nong Khai | Buffalo | Pan Asia |
| O/39/NKPN/00 | Nakhonphanom | Cattle | Pan Asia |
| O/29/NK/00 | Nong Khai | Cattle | Pan Asia |
| O/17/MDH/00 | Mukdahan | Cattle | Pan Asia |
| O/18/UB/00 | Ubon Ratchathani | Cattle | Pan Asia |
| O/32/UB/00 | Ubon Ratchathani | Cattle | Pan Asia |
| O/24/UB/00 | Ubon Ratchathani | Buffalo | Pan Asia |
| O/52/CM/01 | Chiang Mai | Cattle | Pan Asia |
| O/22/CR/00 | Chiang Rai | Cattle | Pan Asia |
| O/41/CR/00 | Chiang Rai | Cattle | Pan Asia |
| O/F48/02/05 | NK | NK | Cathay |
| O/F48/03/05 | NK | NK | Cathay |
| O/189 | NK | NK | Cathay |

SEA = South-East Asia

NK = not known

Table 4 Historical finding of serotype A isolated from Thailand during 1998 to 2003 at Region Reference Laboratory for FMD in South East Asia, Pakchong, Nakhon Ratchasima and Kamphaengsaen Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen, Nakhon Pathom.

| Virus Designation | Geographical origin | Species |
|---|---|---|
| A/56/LP/99 | Lumpun | Cattle |
| A/59/LOEI/99 | Loei | Cattle |
| A/60/LOEI/99 | Loei | Cattle |
| A/61/NK/99 | Nong Khai | Cattle |
| A/62/PSNL/99 | Phitsanulok | Cattle |
| A/63/PSNL/99 | Phitsanulok | Cattle |
| A/71/NRT/98 | Narathiras | Cattle |
| A/72/CYP/00 | Chaiyapum | Cattle |
| A/73/CYP/00 | Chaiyapum | Cattle |
| A/65/UTRD/99 | Uttaradit | Cattle |
| A/F41/09/98 | Kanchanaburi | Cattle |
| A/F41/13/98 | Kanchanaburi | Cattle |
| A/57/PCB/99 | Phetchabun | Cattle |
| A/58/PCB/99 | Phetchabun | Cattle |
| A/78/RCBR/01 | Ratchaburi | Cattle |
| A/67/KCNBR/99 | Kanchanaburi | Cattle |
| A/82/CM/01 | ChaengMai | Cattle |
| A/84/UTRD/01 | Uttaradit | Cattle |
| A75/UDTN/01 | Udon Thani | Cattle |
| A/102/LP/01 | Lampang | NK |
| A/120/SKNK/01 | Sakon Nakhon | Cattle |
| A/114/SSK/02 | Si Sa Ket | Cattle |
| A/66/PSNL/99 | Phitsanulok | Cattle |
| A/83/UTRD/01 | Uttaradit | Buffalo |
| A/77/SSK/01 | Si Sa Ket | Cattle |
| A/79/NK/01 | Nong Khai | Cattle |
| A/81/ANC/01 | Amnat Charoen | Cattle |
| A/F44/11/01 | Nakhon Pathom | Cattle |
| A/F44/14/01 | Nakhon Pathom | Cattle |
| A/115/KLS/02 | Kalasin | Cattle |
| A/117/RCBR/02 | Ratchaburi | Cattle |
| A/121/NKPT/02 | Nakhon Pathom | Swine |

Table 4 (cont'd)

| Virus Designation | Geographical origin | Species |
|---|---|---|
| A/68/YL/98 | Yala | Cattle |
| A/69/YL/98 | Yala | Cattle |
| A/70/YL/98 | Yala | Cattle |
| A/F46/09/03 | Nakhon Pathom | Cattle |
| A/F46/12/03 | Ratchaburi | Swine |
| A/F46/10/03 | Nakhon Pathom | Cattle |
| A/F46/07/03 | Ratchaburi | Cattle |
| A/F46/08/03 | Ratchaburi | Swine |
| A/118/87 | Saraburi | Cattle |
| A/92/22A | NK | NK |
| A/119/NKPT/02 | Nakhon Pathom | Cattle |
| A/116/SK/02 | Songkhla | Cattle |
| A/118/NKPT/02 | Nakhon Pathom | Cattle |
| A/F41/01/98 | Nakhon Pathom | Cattle |
| A/100/SKNK/97 | Sakon Nakhon | NK |
| A/74/MRS/00 | Maha Sarakham | Cattle |
| A/80/KRS/01 | Kalasin | Cattle |

NK = not known

Table 5 History of serotype O obtained from GenBank database.

| Virus Designation | Geographical origin | Species | Topotype | Database accession no. | Reference |
|---|---|---|---|---|---|
| O/Manisa/TUR/96 | Manisa, Turkey | Cattle | ME-SA | AJ251477 | Samuel and Knowles (2001) |
| O/THI/4/99 | Mae Hong Son, Thailand | Cattle | SEA | AJ303536 | Samuel and Knowles (2001) |
| O/THI/1/92 | Narathiwai, Thailand | NK | SEA | AJ303532 | Samuel and Knowles (2001) |
| O/VIT/7/97 | Har,Yrongpa, Gialai,Vietnam | Cattle | SEA | AJ296328 | Samuel and Knowles (2001) |
| O/CAM/2/98 | Kampong, Spey, Cambodia | Cattle | SEA | AJ294909 | Samuel and Knowles (2001) |
| O/VIT/2/97 | Vietnam | Cattle | SEA | AJ294929 | Knowles, *et al.* (2001) |
| O/CAM/1/98 | Cambodia | Swine | SEA | AJ294908 | Knowles, *et al.* (2001) |
| O/CAM/4/2000 | Cambodia | NK | Pan Asia | AJ318829 | Knowles, *et.al.,* 2005 |
| O/MAY/2/2000 | Malaysia | NK | Pan Asia | AJ318846 | Knowles, *et.al.,* 2005 |
| O/JPN/2000 | Japan | NK | Pan Asia | AB050978 | Knowles, *et.al.,* 2005 |
| O/SKR/2000 | South Korea | NK | Pan Asia | AF428246 | Kweon *et al.,* 2002 |
| O/LAO/2/2000 | Loas | NK | Pan Asia | AJ318844 | Knowles, *et.al.,* 2005 |
| O/1734/RUS/2000 | Russia | NK | Pan Asia | AJ318850 | Knowles, *et.al.,* 2005 |
| O/CAM/2/2000 | Cambodia | NK | Pan Asia | AJ318828 | Knowles, *et.al.,* 2005 |
| O/TAW/81/97 | I-lan, Taiwan POC | Swine | Cathay | AJ296321 | Samuel and Knowles (2001) |
| O/GD/CHA/86 | Guangdong, P.R. China | Swine | Cathay | AJ131468 | Samuel and Knowles (2001) |
| O/KEN/83/79 | Mweiga, Kenya | Cattle | EA | AJ303511 | Samuel and Knowles (2001) |
| O/UGA/5/96 | Mbarara, Uganda | Cattle | EA | AJ296327 | Samuel and Knowles (2001) |
| O/CIV/8/99 | Cole d'lvoire | Cattle | WA | AJ303485 | Samuel and Knowles (2001) |
| O/GHA/5/93 | Kintampo, Ghana | Cattle | WA | AJ303488 | Samuel and Knowles (2001) |
| O/1K | Kaufbeuren, F.R. Germany | Cattle | Euro-SA | X00871 | Forss *et.al.(*1984) |
| O/Indonesia/83 | Indonesia | NK | ISA | AJ004683 | Tsai. *et.al.* (2000) |
| O/TAI/1/80 | Thailand | NK | SEA | AJ004681 | Huang *et al.,* (2001) |
| O/MYA/5/99 | Myanmar | Cattle | SEA | DQ164926 | Knowles, *et.al.,* 2005 |
| O/MAY/3/2001 | Malaysia | Cattle | SEA | DQ164920 | Knowles, *et.al.,* 2005 |
| O/MYA/7/98 | Myanmar | Cattle | SEA | DQ164925 | Knowles, *et.al.,* 2005 |
| O/TAI/3/2003 | Thailand | Swine | SEA | DQ164981 | Knowles, *et.al.,* 2005 |
| O/MYA/7/2002 | Myanmar | Swine | SEA | DQ164928 | Knowles, *et.al.,* 2005 |
| O/MAY/5/2001 | Malaysia | Cattle | SEA | DQ164921 | Knowles, *et.al.,* 2005 |
| O/MAY/6/2001 | Malaysia | Cattle | SEA | DQ164922 | Knowles, *et.al.,* 2005 |
| O/TAI/8/99 | Thailand | Cattle | SEA | DQ164977 | Knowles, *et.al.,* 2005 |
| O/LAO/4/98 | Laos | Buffalo | SEA | DQ164906 | Knowles, *et.al.,* 2005 |
| O/MAY/1/2002 | Malaysia | Cattle | SEA | DQ164923 | Knowles, *et.al.,* 2005 |
| O/CAM/3/98 | Cambodia | Cattle | SEA | AJ294910 | Knowles, *et.al.,* 2005 |
| O/VIT/7/97 | Vietnam | NK | SEA | AJ296328 | Knowles, *et.al.,* 2005 |

Table 5 (cont'd)

| Virus Designation | Geographical origin | Species | Topotype | Database accession no. | Reference |
|---|---|---|---|---|---|
| O/VIT/2/97 | Vietnam | Cattle | SEA | AJ294929 | Knowles, *et.al.*, 2005 |
| O/MYA/13/89 | Myanmar | Cattle | SEA | DQ164924 | Knowles, *et.al.*, 2005 |
| O/MYA/2/2000 | Myanmar | Cattle | SEA | DQ164927 | Knowles, *et.al.*, 2005 |
| O/PHI/8/2004 | Philippines | Swine | Cathay | DQ164962 | Knowles, *et.al.*, 2005 |
| O/PHI/11/2004 | Philippines | Swine | Cathay | DQ164965 | Knowles, *et.al.*, 2005 |
| O/PHI/3/2004 | Philippines | Swine | Cathay | DQ164957 | Knowles, *et.al.*, 2005 |
| O/PHI/4/2004 | Philippines | Swine | Cathay | DQ164958 | Knowles, *et.al.*, 2005 |
| O/PHI/6/2004 | Philippines | Swine | Cathay | DQ164960 | Knowles, *et.al.*, 2005 |
| O/PHI/1/2004 | Philippines | Swine | Cathay | DQ164955 | Knowles, *et.al.*, 2005 |
| O/PHI/21/2003 | Philippines | Swine | Cathay | DQ164953 | Knowles, *et.al.*, 2005 |
| O/PHI/10/2003 | Philippines | Swine | Cathay | DQ164951 | Knowles, *et.al.*, 2005 |
| O/PHI/13/2000 | Philippines | Swine | Cathay | DQ164948 | Knowles, *et.al.*, 2005 |
| O/PHI/17/2003 | Philippines | Swine | Cathay | DQ164952 | Knowles, *et.al.*, 2005 |
| O/PHI/5/2000 | Philippines | Swine | Cathay | DQ164947 | Knowles, *et.al.*, 2005 |
| O/PHI/7/96 | Philippines | Swine | Cathay | AJ294926 | Knowles, *et.al.*, 2005 |
| O/PHI/5/95 | Philippines | Swine | Cathay | DQ164946 | Knowles, *et.al.*, 2005 |
| O/HKN/12/91 | Hong Kong | Swine | Cathay | AJ294921 | Knowles, *et.al.*, 2005 |
| O/TAW/4/99 | Taiwan | Swine | Cathay | AJ294928 | Knowles, *et.al.*, 2005 |
| O/VIT/3/97 | Vietnam | Swine | Cathay | AJ294930 | Knowles, *et.al.*, 2005 |
| O/HKN/21/70 | Hong Kong | Swine | Cathay | AJ294911 | Knowles, *et.al.*, 2005 |
| O/HKN/6/83 | Hong Kong | Cattle | Cathay | AJ294919 | Knowles, *et.al.*, 2005 |
| O/VIT/2/2004 | Vietnam | Swine | Cathay | DQ165033 | Knowles, *et.al.*, 2005 |
| O/VIT/13/2002 | Vietnam | NK | Cathay | DQ165025 | Knowles, *et.al.*, 2005 |
| O/HKN/20/96 | Hong Kong | Cattle | Cathay | AJ294924 | Knowles, *et.al.*, 2005 |
| O/HKN/4/2001 | Hong Kong | NK | Cathay | DQ164875 | Knowles, *et.al.*, 2005 |
| O/HKN/19/2001 | Hong Kong | Swine | Cathay | DQ164876 | Knowles, *et.al.*, 2005 |
| O/HKN/2/2003 | Hong Kong | Swine | Cathay | DQ164879 | Knowles, *et.al.*, 2005 |
| O/HKN/3/2004 | Hong Kong | Swine | Cathay | DQ164880 | Knowles, *et.al.*, 2005 |
| O/HKN/10/2004 | Hong Kong | Swine | Cathay | DQ164887 | Knowles, *et.al.*, 2005 |

Europe-South America (Euro-SA), Middle East- South Asia (ME-SA), South-East Asia (SEA), Cathay (an ancient and poetic name for China and east Tartary), West Africa (WA), East Africa (EA), Indonesia (ISA)

NK = not known

Table 6 History of serotype A obtained from GenBank database

| Virus Designation | Geographical origin | Species | Database accession no. | Genotypes |
|---|---|---|---|---|
| IND/302/88 | India | Cattle | AF390641 | IV |
| IND/160/90 | India | Cattle | AF390608 | IV |
| A22/Azerbaijan/65 | Russia | NK | X74812 | IV |
| NE/EL/60 | China | NK | AJ131663 | III |
| GS/LX/62 | China | NK | AJ131666 | III |
| XJ/KT/58 | China | NK | AJ131665 | III |
| IND/408/97 | India | Cattle | AF390647 | VII |
| IND/174/2000 | India | Cattle | AF390617 | VII |
| IND/96/96 | India | Cattle | AF390673 | VII |
| IND/55/86 | India | Cattle | AF390655 | I |
| IND/77/86 | India | Cattle | AF390663 | I |
| A10/Holland | Holland | NK | M20715 | I |
| A10-61 | NK | NK | X00429 | I |
| A12-119b | NK | NK | J02187 | I |
| A5/Spain/86 | Spain | NK | M72587 | I |
| A/Madrid(E)/1983 | Madrid | NK | M16084 | I |
| A27/Colombia/76 | Colombia | NK | K03341 | I |
| A/Argentina/79 | Argentina | NK | K03345 | V |
| A/Venceslau/Brazil/76 | Brazil | NK | K03344 | V |
| A24/Brazil/55 | Brazil | NK | K03340 | V |
| A/Argentina/87 | Argentina | NK | U62255 | II |

NK = not known

Source: Tosh (2002)

Table 7 History of serotype Asia1 obtained from GenBank database.

| Virus Designation | Geographical origin | Species | Database accession no. | Lineages |
|---|---|---|---|---|
| Asia1/PAK/1/54 | Pakistan | NK | AJ251478 | I |
| Asia1/TAI/1/98 | Thailand | NK | DQ121129 | II |
| Asia1/LEB/83 | Lebanon | NK | AJ294931 | III |
| Asia1/China | China | NK | AF241566 | IV |
| IND/24/95 | India | Cattle | AF392933 | V |
| IND/305/94 | India | Cattle | AF392936 | V |
| IND/63/72 | India | Cattle | AF292106 | V |
| IND/390/97 | India | Cattle | AF392940 | VI |
| IND/397/97 | India | Cattle | AF392942 | VI |
| IND/278/99 | India | Cattle | AF392934 | VI |
| IND/247/92 | India | Cattle | AF392932 | VI |
| IND/396/97 | India | Cattle | AF392941 | VI |

NK = not known

Source: Sanyal (2004)

## 2. Synthetic Peptide Vaccine

### 2.1 Vaccine

The synthetic peptide vaccine, designated as UBI FMDV Th Formulation1 was used in this study incorporated with United Biomefical Inc. The synthetic peptide vaccine in this study was constructed that increased maximum immunogenicity and broad cross reactivity against antigenic variant by Wang *et al.*, (2002). It consisted of (1) consensus amino acid residues of the G-H loop domain at position 129-169 for broad cross reactivity which elicited more broadly neutralizing antibodies against the Manisa and Campos isolates than a conventional O1 Taiwan killed virus vaccine, (2) disulphide bond formed between the cysteine residues at position 134 and 158, which cyclisation occurred by disulphide bond stabilized the presentation of a flexible G-H loop-like structure, (3) the contiguous flanking sequences on both the amino and carboxy termini and (4) promiscuous Th epitopes from measles virus outside VP1. The synthetic peptide vaccine was in water in oil emulsions mixed 1:1 with Montanide ISA 51 (Seppic, Paris). Positive group was immunized by commercial vaccine (trivalent), and UBI FMDV Th formulation 3 (without antigen) was used in control group.

### 2.2 Animals and Vaccination

Pigs were divided into 3 groups. Each group consisted of 8 weeks age of two females and two males. First group was immunized with UBI FMDV Th Formulation1. Second group was induced by commercial vaccine and the last one (control group) was induced by UBI FMDV Th formulation 3. Each group was immunized at day 0 and 30. The pigs were bled on days of vaccination and 2, 3 and 4 weeks after 2$^{nd}$ vaccination. Sera were collected and analyzed by serological tests.

### 2.3 Challenge Procedure

The pigs were challenged with $10^7$ $TCID_{50}$ of field isolate propagated in BHK21 at 4 weeks after $2^{nd}$ vaccination. Blood was collected after 2 weeks of challenge and used for serological tests. The FMDV challenge procedure was manipulated by veterinarian of Foot and Mouth Disease Center, Pakchong, Nakhon Ratchasima.


**Figure 5** The structure of synthetic peptide vaccine. The consensus peptide of position 129-169 of serotype O was showed at the lower line. The RGD (underline) was receptor on G-H loop and the position 158 (*) residue was modified to cysteine. T cell helper epitope (upper line) was linked through an ε–Lysine linker to the consensus site. The numbers were showed position of consensus peptide.

Source: Wang (2002).

### 2.4 Serological Tests

Virus-neutralizing activity was followed Foot and Mouth Disease Center's protocol. Briefly, sera were heated at 56°C for 30 minutes to inactivate complements then diluted 50 µl of inactivated sera to serial three-fold dilutions (1:3-1:178) in flat-bottomed microplate and neutralized with 50 µl of 320 $TCID_{50}$/ml of reference viruses provided from Foot and Mouth Disease Center for 1 hour at 37°C in 5% $CO_2$. Subsequently, 150 µl secondary lamb kidney cell were added in all wells and incubated for 72 hours at 37°C in 5% $CO_2$. The cytopathic effect (CPE) was observed under inverted microscope. The $\log_{10}$ of SN titer was calculated by Karber method (Karber, 1931)

Commercial ELISA test kits, (UBI® FMDV VP1 ELA and UBI® FMDV NS EIA), were used to evaluate the antibody response. They were performed according to the manufacturer's instructions (Appendix C)

## 3. 3AB Enzyme Linked Immunosorbent Assay

The PCR technique and gene cloning of 3AB recombinant protein were reported by Lertwatcharasarakul (2000). Briefly, the 3AB gene of serotype A was synthesized for cDNA and PCR product. The PCR product was cloned into PCRII (invitrogen®) and proved by nucleotide sequencing (Appendix Figure D1). It was subcloned into E*coR*I site of pET28A (Novagen®) and B*amH*I site of pQE30 (Qiagen®), respectively. The recombinant 3AB protein was produced by *E.coli* strain M15, and an antigenic specificity was confirmed by western blotting and dot blotting.

### 3.1 Purification of Recombinant 3AB Protein

After a single colony of recombinant *E.coli* was cultured overnight in 10 ml of Luria Bertani (LB) broth containing 100 μg/ml ampicillin in incubator shaker. The overnight culture was diluted 1:50 in 200 ml fresh LB containing 100 μg /ml ampicillin. The dilution was preformed 3 flasks in preparation of 3AB antigen, control antigen and sample diluents. The 1mM isopropyl-$\beta$-D thiogalactopyranoside (IPTG) was added in 3AB antigen flask at 3 hours after subculture initiation. The cells were collected after 7 hours of culture by centrifuged at 5,000 rpm for 5 minutes and washed once with 5 ml of PBS.

The pellets of 3AB antigen and control antigen were performed by partial purification. It was done by dissolving the cell pellets in 4ml of 4M, 6M and 8M urea, respectively. The insoluble materials of each urea concentrations were broken with sonicator on ice at 5 amplitudes for 10 second and recovered by centrifuge for 30 minutes at 10,000 rpm. Sequentially, the remained insoluble materials were dissolved in 4 ml of 1% SDS and glycerol was added to a final concentration of 50% before stored at -20°C.

For sample diluent preparation, the pellet from the third flask was resuspended in 4 ml PBS then was boiled for 5 minutes and broken on ice for 4 times using sonicator at 5 amplitude for 30 seconds on ice. It was frozen in -20°C until use.

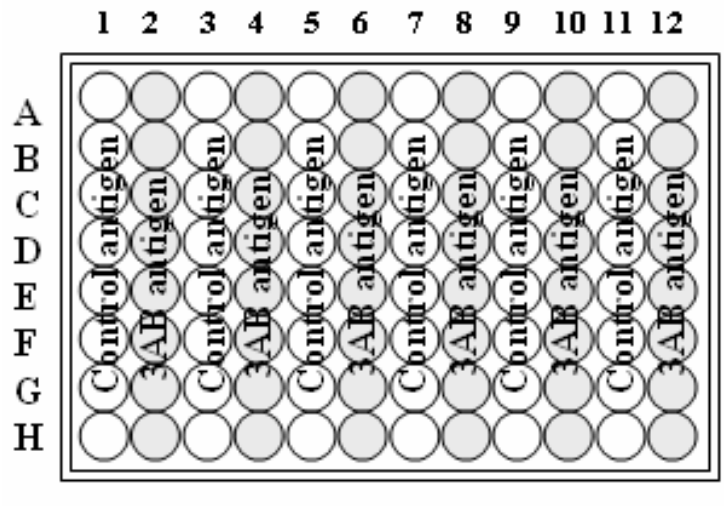
### 3.2 Indirect Enzyme Linked Immunosorbent Assay (iELISA)

1:400 of 3AB antigen and control antigen in coating buffer (Appendix A) was bound at 4°C overnight in an odd number column and an even number column of antigen-coated microplate (Maxisorp, nunc™), respectively (Figure 6a). After the antigen-coated microplate was washed four times with PBST (PBS including 0.05% Tween20), the antigen-coated microplate was blocked for 1 hour with blocking buffer (Appendix A). Then, the antigen-coated microplates were tapped firmly and dried completely at room temperature. The antigen-coated microplate was kept in dark and cool place (4°C).

The sample diluent was fresh prepared in 1:100 of 1X PBST for serum dilution before test. Test sera and control sera were diluted 1:100 with sample diluent in dilution plate and incubated 37°C for 1 hour in incubator. 100 µl of each diluted test sera and control sera were added into antigen-coated microplate followed format (Figure 6b) and incubated at 37°C for 1 hour. It was washed four times with PBST and tapped firmly on absorbent papers and 100 µl of the 1:20000 recombinant protein G conjugated HRP (Zymed®) in 1X PBST was added to all wells of the antigen-coated microplate and incubated at 37°C for 30 minutes. It was washed four times with PBST and then tapped on absorbent papers and 100 µl of TMB substrate (Zymed®) was added and incubated in the dark for 10 minutes at 37°C. Then, the reaction was stopped with 100 µl of 0.01% SDS. The optical density (OD) reading was measured with an automatic microplate reader at a wavelength of 650 nm. The test was designated to KU-ELISA3AB. The sample/positive (S/P) ratio was calculated according to the formula:

$$\text{S/P ratio} = \frac{\text{(3AB antigen – control antigen of test serum)}}{\text{(3AB antigen – control antigen of positive control serum)}}$$

**a.**




**b.**

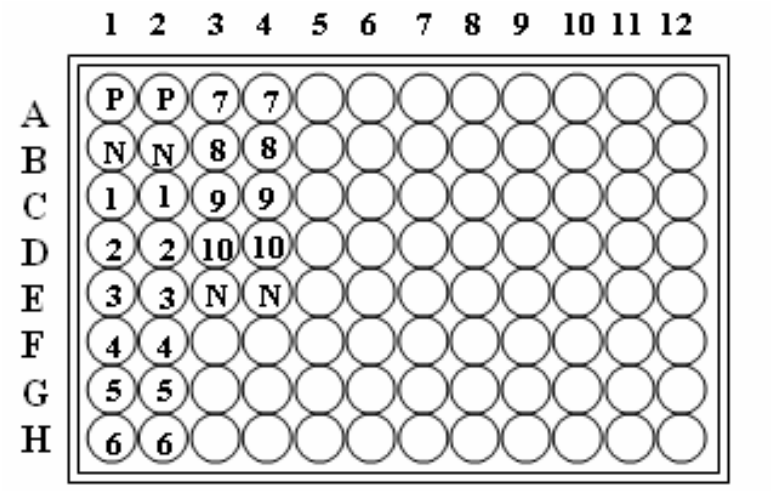



Figure 6 Format of the control and 3AB antigen coating in antigen-coated microplate (a) and sample distribution (b). Positive (P), Negative (N), Test samples (1,2,3…N)

### 3.3 Serum Samples for the KU-ELISA 3AB Evaluation

Serum samples for the establishment of cut off values were collected from 195 imported swine, and 144 sera of vaccinated-swine were collected from sows in FMD-free swine farms based on the vaccination program. One hundred and twenty one bovine sera of FMD free farm were evaluated for the cut off value.

One hundred and two of infected swine and seventy one of infected bovine from naturally FMD outbreak farms were evaluated for sensitivity test

Seventy nine of infected swine sera were compared with two commercial test kit, CHEKIT® FMD-3ABC po (Bommeli Diagnostic, Switzerland), and UBI® FMDV NS NIA (United Biomefical Inc., New York, USA) designated as ELISA-A and B, respectively. Seventy five of bovine classified into two groups, sixty four infected-bovine sera collected after 14 months of FMD outbreak and eleven calves born from the first group aging between 7 to 13 months were compared with Ceditest® FMDV NS test kit (Lelystad, The Netherlands), designated as ELISA-C. The commercial test kits were performed following the manufacturer's descriptions (Appendix C).

### 3.4 Evaluation of Assay Repeatability and Agreement between Kit

Evaluations of assay repeatability within and between run tests were performed as proposed by Jacobson (1998). Four replicating values of each serum samples were assigned in the same plate for intra-assay (within-same plate) repeatability and run in different plates on different occasions for inter-assay (between run) repeatability. Mean of S/P ratio, standard deviation (SD), and coefficient of variant (CV) of four replicating values of each test were calculated.

The level of agreement between ELISA was determined by the kappa statistic (Thrusfield, 1995). The agreement was classified by values of kappa statistics into almost perfect (0.81 or higher), substantial (0.61-0.80), moderate (0.41-0.60), fair (0.21-0.40) and slight (0-0.20).

## 4. 3AB Monoclonal Antibodies

### 4.1 3AB Antigen Preparation and Mouse Immunization

3AB antigen, which was partially purified for KU-ELISA 3AB, was separated by 10% SDS-PAGE and stained with copper stain (Appendix A). Specific protein band compared with molecular protein marker was cut by blade and ground by glass bar in 500 µl 0.1% SDS. The gel was sonicated 3 times at 5 amplitude 30 second on ice. The polyacrylamine gel was separated by centrifuge at 10,000 rpm for 5 minutes, and supernatant was transferred to fresh microtube. The protein precipitation was added 4 volumes (V/V) of cool acetone incubated overnight in -80 °C. Protein collection was approached by centrifuge at 15000 rpm for 15 minutes. Acetone was discarded by gently aspiration and completely dried at room temperature. 50 µg protein in 0.5 ml of PBS was mixed with an equal volume of freund's complete adjuvant. The injection was done in mouse by intraperitoneal route. Subsequently, the injection dose of 25 µg protein with freund's incomplete adjuvant was used at 2 weeks interval. Mouse was bled after immunization every week. The antibody titer was measured by KU-ELISA 3AB test kit followed descriptive methods, but 1:2000 of goat anti-mouse IgG (H+L) conjugated HRP (Zymed®) in 1X PBST was used for 2nd antibody instead of protein G usage. Result interpretation was compared with the serum before immunization.

### 4.2 Myeloma Cell

Myeloma strain P3-X63-Ag8.653 was cultured in CM medium (Appendix A) including 10% fetal bovine serum (HyClon® ), which was passaged every 3 days for maintenance.

### 4.3 Feeder Cell Preparation

The thymus was used for feeder cell preparation which was collected from 6 weeks mouse by aseptic technique and was ground with sterile glass slide. The cells were washed 3 times of sterile CM medium and recovered by centrifuge at 10,000 rpm for 5 minutes. The cells were resuspended with HT medium (Appendix A), and 100µl of cell suspension was added into 96 well plate incubated at 37°C and 5% $CO_2$

### 4.4 Cell Fusion and Limiting

Before 3 days cell fusion, the mouse was booster with 25 µg of 3AB protein with freund's incomplete adjuvant. The spleen was collected with aseptic technique and ground by sterile glass slide in 200 ml CM medium. The spleen cells were washed three times with CM medium and centrifuged at 1,500 rpm for 5 minutes. The myeloma cells were collected with cell scalper and washed 3 times with CM medium then mixed with spleen cells at a ratio 1:2 (myeloma cells: spleen cells) number of cells. Subsequently, they were added with 1 ml polyethylene glycol (PEG) 1500 incubated at 37°C for 5 minutes and 5 ml CM medium for 5 minutes. The cells were resuspended with 40 ml HAT medium (Appendix A) and added 100 µl each well of 96 well plate containing feeder cells.

HAT medium was changed every few days. Hybridoma cells were initially screened by KU-ELISA 3AB test kit after 2 weeks of culture. The positive wells were performed of cell limiting. They were counted by hemocytometer chamber and resuspended with HT medium (Appendix A) to 0.5 cell per well. Antibodies against 3AB protein were initially tested by KU-ELISA 3AB after 2 weeks culture. The positive wells were used for second cell limiting and tested ELISA. Supernatant of hybridoma was evaluated by UBI® FMDV NS NIA (United Biomefical Inc., New York, USA), KU-ELISA 3AB test kit, Immunoperoxidase monolayer assay (IPMA) and antibody isotype classification.

### 4.5 Antibody Isotype

Isotyping of antibodies was used Mouse MonoAb ID Kit (HRP) (ZYMED®) Procedure was followed by manufactory's description (Appendix C)

### 4.6 Evaluation of Specific Antibody by ELISA

Antibody Specificity were evaluated by UBI® FMDV NS NIA (United Biomefical Inc., New York, USA) (Appendix C) and KU-ELISA 3AB test kit. Both protocols were modified by changing $2^{nd}$ antibody from protein G to 1:2000 of goat anti-mouse IgG (H+L) conjugated HRP (Zymed®) in 1X PBST.

### 4.7 Immuno Peroxidase Monolayer Assay (IPMA)

Propagated virus in Baby Hamster Kidney (BHK) 21 cells in 96 well culture plate at 24 hour was used to test. Medium was discarded and washed the cells with 200 µl 1X PBST. Proteins were fixed with 100 µl 4% formalin for 10 min before washed 3 times with PBST. 100 µl of 0.5% saponin solution were added and incubated for 10 min and washed 3 times with 1X PBST. The culture plate was blocked with 1% skim milk at 37°C for 1 hour, then 100 µl of 1:50 monoclonal antibodies in 1XPBST were added and incubated at 37°C for 1 hour. Goat anti mouse (H+L) conjugated HRP (Zymed®) were prepared at 1:1000 in PBS, then added 100 µl into each well. 100 µl DAB substrate solution (Appendix A) was added for 15 min and stopped reaction by washing with tap water. The results were investigated by inverted microscope, which positive results appeared in brown insoluble material into infected cell.

# RESULTS

## 1. Phylogenetic Tree

For preliminary study of phylogenetic tree, one hundred ninety base pairs (nucleotide 361 to 550 of VP1) (Figure 7) of three sequences of Thailand isolates and ten sequences from GenBank were used to compare with complete VP1 gene and nucleotide 469 to 639. The results showed the similarity of phylogenetic tree pattern (Figure 8), identity and divergence percentage (Table 8). Therefore, the nucleotide 361 to 550 of VP1 gene was used to construct the phylogenetic tree among virus isolates in Thailand.

### 1.1 Serotype O

Sixty four isolates of FMDV serotype O isolated during 1998 to 2005 were used for investigation of genetic variant based on partial C-terminal of VP1 (190 bp). The comparative nucleotide sequence of viruses isolated in Thailand from different of geographical areas were found to be located in SEA topotype, Pan-Asia strain in ME-SA topotype and Cathay topotype (Figure 9).

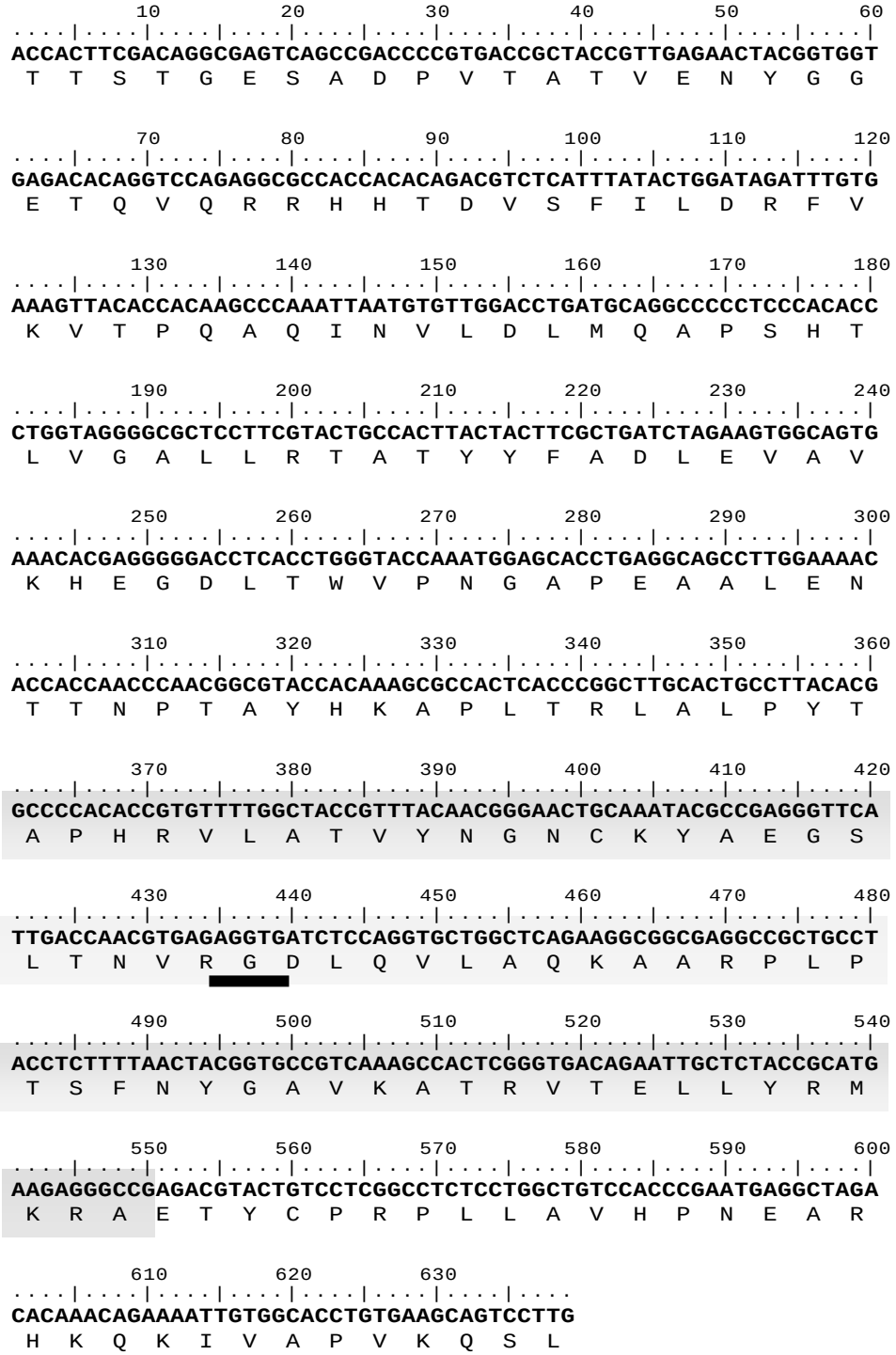
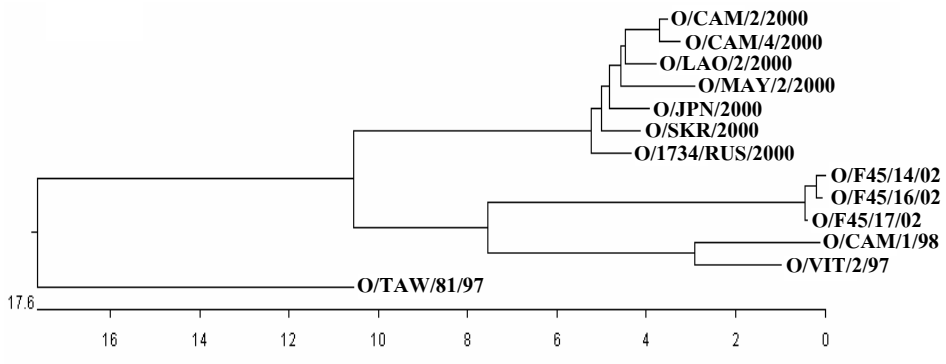
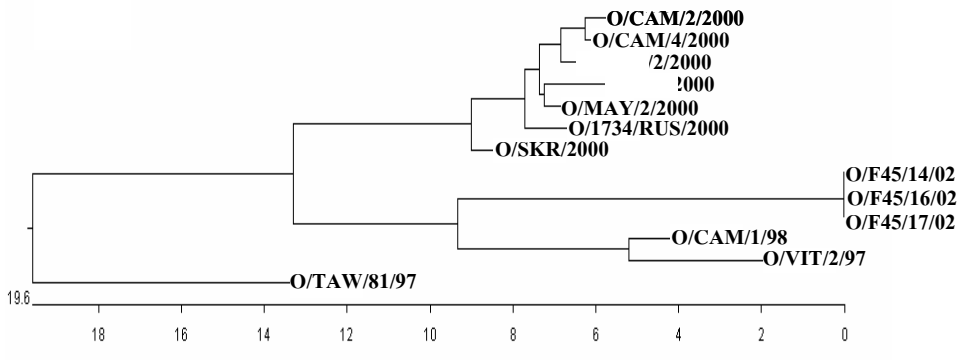



Figure 7 Nucleotide sequence and deduced amino acid of whole VP1 gene based on O/F45/14/02. Black underline was represented motif of amino acid (RGD). Numbers indicated as the position nucleotide sequence. Amino acid residues were indicated below the nucleotide sequence by their single letter codes. Gray box indicated the nucleotide sequence from 361 to 550 bp.

a.




b.




c.

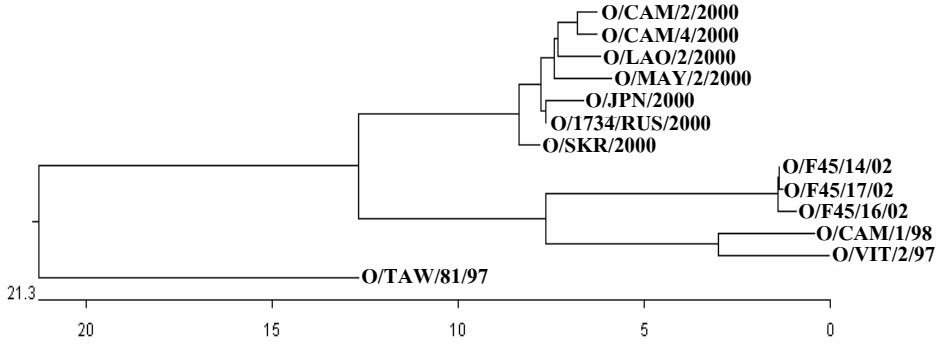



Figure 8 Comparison of phylogenetic tree based on complete gene (a) nucleotide 469 to 639 (b) and nucleotide 361 to 550 (c) of VP1 gene.

**a**

Percent Identity

Divergence

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 97.7 | 97.0 | 94.5 | 96.4 | 96.2 | 96.1 | 82.3 | 82.5 | 82.8 | 81.5 | 82.6 | 77.3 | 1 |
| 2 | 0.6 | | 96.6 | 93.6 | 95.9 | 95.8 | 95.9 | 82.5 | 82.6 | 82.9 | 81.7 | 82.8 | 76.8 | 2 |
| 3 | 1.6 | 1.9 | | 96.1 | 98.1 | 98.0 | 97.8 | 83.9 | 83.9 | 84.4 | 83.1 | 83.7 | 78.4 | 3 |
| 4 | 2.6 | 3.0 | 2.4 | | 95.6 | 95.6 | 95.3 | 80.9 | 80.9 | 81.4 | 80.9 | 82.0 | 76.1 | 4 |
| 5 | 2.3 | 2.6 | 1.9 | 2.8 | | 98.3 | 97.8 | 84.0 | 84.0 | 84.5 | 82.9 | 83.6 | 78.6 | 5 |
| 6 | 2.4 | 2.8 | 2.1 | 2.9 | 1.8 | | 97.7 | 85.0 | 85.0 | 85.4 | 83.9 | 85.0 | 79.3 | 6 |
| 7 | 2.6 | 2.6 | 2.2 | 3.1 | 2.2 | 2.4 | | 84.4 | 84.4 | 84.8 | 83.9 | 85.3 | 78.9 | 7 |
| 8 | 17.3 | 17.3 | 17.5 | 18.2 | 17.1 | 16.2 | 16.8 | | 99.7 | 99.5 | 85.4 | 85.6 | 75.3 | 8 |
| 9 | 17.1 | 17.1 | 17.5 | 18.2 | 17.1 | 16.2 | 16.8 | 0.3 | | 99.5 | 85.6 | 85.8 | 75.1 | 9 |
| 10 | 16.9 | 16.9 | 17.1 | 17.8 | 16.7 | 15.8 | 16.4 | 0.5 | 0.5 | | 85.8 | 85.9 | 75.6 | 10 |
| 11 | 17.5 | 17.5 | 17.9 | 18.0 | 17.9 | 16.6 | 16.6 | 14.6 | 14.6 | 14.4 | | 95.1 | 76.7 | 11 |
| 12 | 16.5 | 16.5 | 16.9 | 17.2 | 17.3 | 15.6 | 15.2 | 14.3 | 14.3 | 14.1 | 4.7 | | 77.0 | 12 |
| 13 | 20.6 | 21.1 | 21.2 | 22.2 | 21.0 | 20.3 | 20.5 | 25.9 | 25.8 | 25.4 | 23.3 | 22.9 | | 13 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |

O/CAM/2/2000
O/CAM/4/2000
O/LAO/2/2000
O/MAY/2/2000
O/JPN/2000
O/SKR/2000
O/1734/RUS/2000
O/F45/14/2002
O/F45/16/2002
O/F45/17/2002
O/CAM/1/98
O/VIT/2/97
O/TAW/81/97

**b**

Percent Identity

Divergence

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 97.9 | 96.8 | 96.3 | 96.3 | 97.4 | 96.3 | 82.1 | 82.0 | 82.1 | 80.5 | 80.5 | 74.2 | 1 |
| 2 | 1.1 | | 96.8 | 96.3 | 96.3 | 97.4 | 96.3 | 82.6 | 82.5 | 82.6 | 81.1 | 81.1 | 73.7 | 2 |
| 3 | 2.2 | 2.2 | | 97.4 | 97.4 | 98.4 | 97.4 | 83.7 | 83.6 | 83.2 | 81.1 | 80.5 | 74.7 | 3 |
| 4 | 2.7 | 2.7 | 2.7 | | 96.8 | 97.9 | 96.8 | 83.2 | 83.1 | 82.6 | 82.1 | 82.1 | 74.7 | 4 |
| 5 | 2.7 | 2.7 | 2.7 | 3.3 | | 98.9 | 97.9 | 83.7 | 83.6 | 83.2 | 81.1 | 81.1 | 75.8 | 5 |
| 6 | 1.6 | 1.6 | 1.6 | 2.2 | 1.1 | | 98.9 | 83.7 | 83.6 | 83.2 | 82.1 | 82.1 | 75.8 | 6 |
| 7 | 2.7 | 2.7 | 2.7 | 3.3 | 2.2 | 1.1 | | 85.3 | 85.2 | 84.7 | 83.2 | 83.2 | 76.8 | 7 |
| 8 | 17.8 | 17.8 | 17.5 | 16.8 | 16.8 | 16.8 | 15.4 | | 100.0 | 99.5 | 87.9 | 85.3 | 71.1 | 8 |
| 9 | 17.9 | 17.9 | 17.6 | 16.9 | 16.9 | 16.9 | 15.5 | 0.0 | | 99.5 | 87.8 | 85.2 | 70.9 | 9 |
| 10 | 17.8 | 17.8 | 18.2 | 17.5 | 17.5 | 17.5 | 16.1 | 0.5 | 0.5 | | 87.4 | 84.7 | 70.0 | 10 |
| 11 | 19.2 | 19.2 | 20.4 | 18.2 | 19.7 | 18.2 | 16.8 | 13.0 | 13.0 | 13.6 | | 94.2 | 72.1 | 11 |
| 12 | 19.2 | 19.2 | 20.4 | 18.2 | 19.7 | 18.2 | 16.8 | 14.3 | 14.4 | 14.9 | 5.6 | | 71.1 | 12 |
| 13 | 23.0 | 23.0 | 24.2 | 24.2 | 22.7 | 22.7 | 22.7 | 30.5 | 30.7 | 31.3 | 26.3 | 28.7 | | 13 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |

O/CAM/2/2000
O/CAM/4/2000
O/LAO/2/2000
O/MAY/2/2000
O/JPN/2000
O/1734/RUS/2000
O/SKR/2000
O/F45/14/2002
O/F45/17/2002
O/F45/16/2002
O/CAM/1/98
O/VIT/2/97
O/TAW/81/97

**c**

Percent Identity

Divergence

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 94.7 | 94.2 | 92.4 | 93.6 | 92.4 | 91.8 | 76.6 | 76.6 | 76.6 | 78.9 | 76.0 | 76.0 | 1 |
| 2 | 1.2 | | 94.7 | 93.0 | 94.2 | 94.2 | 92.4 | 78.9 | 78.9 | 78.9 | 81.3 | 78.4 | 77.2 | 2 |
| 3 | 1.8 | 1.8 | | 97.1 | 98.2 | 97.1 | 96.5 | 83.0 | 83.0 | 83.0 | 84.8 | 81.9 | 80.7 | 3 |
| 4 | 3.8 | 3.7 | 3.0 | | 97.7 | 96.5 | 97.1 | 81.9 | 81.9 | 81.9 | 84.2 | 81.3 | 78.4 | 4 |
| 5 | 2.5 | 2.5 | 1.8 | 2.4 | | 97.7 | 97.1 | 82.5 | 82.5 | 82.5 | 85.4 | 83.0 | 80.1 | 5 |
| 6 | 3.7 | 2.5 | 3.0 | 3.6 | 2.4 | | 95.9 | 83.6 | 83.6 | 83.6 | 87.1 | 84.8 | 80.7 | 6 |
| 7 | 4.4 | 4.4 | 3.6 | 3.0 | 3.0 | 4.2 | | 84.2 | 84.2 | 84.2 | 87.1 | 84.8 | 81.9 | 7 |
| 8 | 22.1 | 20.2 | 19.6 | 20.4 | 20.4 | 18.6 | 17.9 | | 100.0 | 100.0 | 84.8 | 83.6 | 73.7 | 8 |
| 9 | 22.1 | 20.2 | 19.6 | 20.4 | 20.4 | 18.6 | 17.9 | 0.0 | | 100.0 | 84.8 | 83.6 | 73.7 | 9 |
| 10 | 22.1 | 20.2 | 19.6 | 20.4 | 20.4 | 18.6 | 17.9 | 0.0 | 0.0 | | 84.8 | 83.6 | 73.7 | 10 |
| 11 | 19.0 | 17.2 | 17.5 | 18.3 | 16.7 | 14.3 | 14.4 | 15.9 | 15.9 | 15.9 | | 95.3 | 81.9 | 11 |
| 12 | 21.6 | 19.7 | 19.9 | 20.8 | 19.1 | 16.6 | 16.7 | 15.9 | 15.9 | 15.9 | 4.9 | | 77.8 | 12 |
| 13 | 19.5 | 19.4 | 19.5 | 22.0 | 19.5 | 19.4 | 17.9 | 29.0 | 29.0 | 29.0 | 18.5 | 21.6 | | 13 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |

O/CAM/2/2000
O/CAM/4/2000
O/LAO/2/2000
O/MAY/2/2000
O/JPN/2000
O/1734/RUS/2000
O/SKR/2000
O/F45/14/2002
O/F45/16/2002
O/F45/17/2002
O/CAM/1/98
O/VIT/2/97
O/TAW/81/97

Table 8 showed the identity and divergence percentage of nucleotide sequences based on complete gene (a), nucleotide 469 to 639 (b) and nucleotide 361 to 552 (c) of VP1 gene

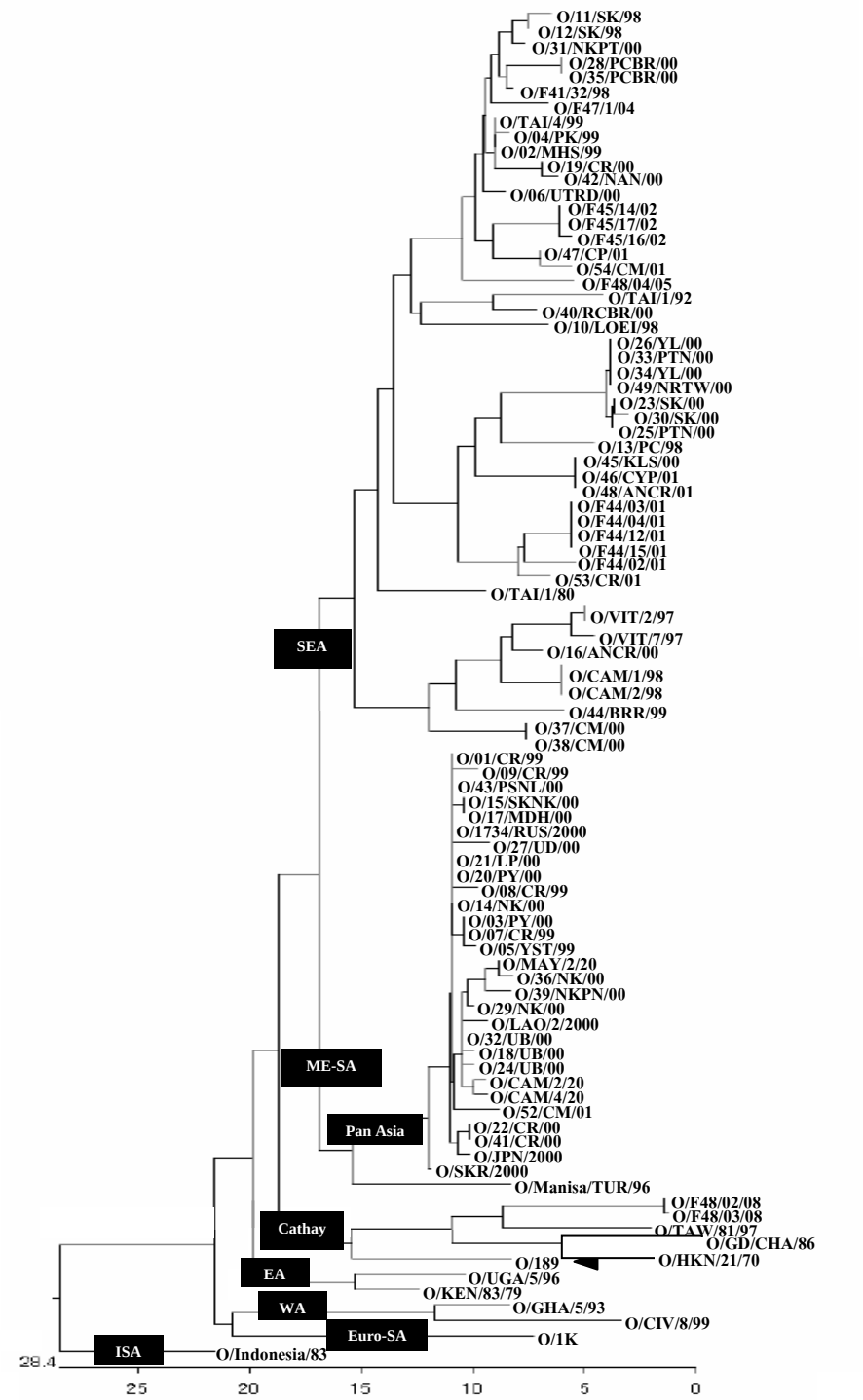



Figure 9 Phylogenetic tree of sixty four isolates of serotype O isolated in Thailand compared with other sequences from GenBank database based on nucleotide 361-550. Black arrow is O/189 that has been used as the seed virus for inactivated vaccine in Thailand. Abbreviation: Europe-South America (Euro-SA), Middle East- South Asia (ME-SA), South-East Asia (SEA), Cathay (an ancient and poetic name for China and east Tartary), West Africa (WA), East Africa (EA), Indonesia (ISA)

The majority of the viruses isolated during 1998 to 2005 were located in SEA topotype. This group had a wide ranged of divergence percentage which can be divided into 3 lineages (I, II and III) based on dendrogram and more than 15% divergence percentage of nucleotide sequences between lineages (Figure 10). The lineage I contained viruses from several parts of Thailand which circulated for a long endemic time in Thailand since 1980 to 2005. Maximum divergence percentage between the Thailand' isolates of this lineage was 14.4% (O/40/RCBR/00 and O/F48/04/05) excluding viruses isolated in 1992 (O/TAI/1/92) and 1980 (O/TAI/1/80) (Samuel and Knowles, 2001). The divergence percentage of both viruses (O/TAI/1/92 and O/TAI/1/80) calculated with the same lineage was ranged from 6.7-16.4% and 10.4-15.9%, respectively. Seventeen viruses isolated during 1998 to 2001 from Thailand were clustered into lineage II and had the maximum divergence percentage among viruses at 12.3%. The last 4 viruses isolated from the Northern and the Northeastern parts of Thailand during 1999 to 2000 were located in the lineage III and the maximum divergence percentage of these isolates was 11.7%. This lineage also contained viruses isolated from Myanmar in 1989, Viet Nam in 1997, Lao in 1998, Cambodia in 1998 and Malaysia in 2002.

The lineage I was subdivided into 6 sublineages (A to F) according to the criteria that the viruses belonging to the same sublineages should have the nucleotide identity more than 95%. Sublineage IA contained viruses isolated mainly during 1998 to 2004 which circulated in the Northern, Southern and Central parts of Thailand and also the viruses that were isolated from Myanmar in 1999 and 2000 and Malaysia in 2001. Sublineage IB consisted of 5 viruses isolated from Thailand during 2001 to 2002, Malaysia in 2001 and Myanmar in 2002. A virus isolate (O/F48/04/05) isolated at the Veterinary Diagnostic Center of the Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen campus in 2005 was belonged to sublineage IC. Sublineage ID contained only one virus (O/10/LOEI/98) isolated from Loei province in 1998. The virus isolated from Ratchaburi province in 2000 was grouped into sublineage IE including a virus (O/TAI/1/92) isolated in 1992 (Samuel and Knowles, 2001). A virus isolated in 1980 (Samuel and Knowles, 2001) was belonged to a last groups (sublineage IF).

Lineage II can be subdivided into 4 sublineages. Sublineage IIA was commonly found to be viruses isolated from the Southern part of Thailand (Narathiwat, Songkhla, Pattani and Yala provinces) in 2000. A virus of sublineage IIB was isolated from Phichit province in 1998. Sublineage IIC was found to be the viruses isolated from the Central (Nakhon Pathom) and the Northern parts of Thailand (Nakhon Sawan and Chiang Rai) in 2001. Sublineage IID was composed of three viruses isolated from the Northeastern part of Thailand (O/46/CYP01, O/48/ANCR/01 and O/45/KLS/00).

Lineage III was subdivided into 3 sublineages. Sublineage IIIA contained viruses isolated from Buriram provinces (O/44/BRR/99) and also from the neighboring countries submitted in GenBank (O/TAI/8/99, O/LAO/4/98 and O/MAY/1/2002). Sublineage IIIB was isolated from Amnat Charoen province (O/16/ANCR/00). Lineage IIIC was comprised two viruses isolated from Chiang Mai in 2001 and one virus isolated from Myanmar in 1989.

Twenty two viruses isolated from the Northern and Northeastern parts of Thailand during 1999 to 2001 were located in Pan- Asia strain of ME-SA topotype. These viruses had the divergence percentage less than 3.8% among the virus isolates in Thailand and obtained from GenBank.

Two viruses isolated (O/F48/02/05 and O/F48/03/05) in 2005 fell in Cathay topotype including the vaccine strain (O/189). The vaccine strain was correlated to the O/HKN/21/70 virus which had 11.6% divergence percentage. Both viruses (O/F48/02/05 and O/F48/03/05) were closely related to the O/VIT/2/2002 virus when compared with other sequences from GenBank database (Figure 11).

The C-terminal region of VP1 of FMDV was the hypervariable region, receptor and major antigenic site and was frequently suffer from the amino acid residues replacement. The consensus of critical amino acid residues (144, 147, 148, 149 and 154) was V, D, L, Q and K amino acid, respectively. However, the replacement of the critical amino acid residues at position 149 from Q to R was observed in one isolate of the sublineage IA and two isolates of Pan Asia

strain. Only one virus isolate of sublineage IIA had an alternative residue at critical position 154 changing from K to R and a virus isolate of sublineage IB (O/54/CM/01) had amino acid substituted at position 144 from V to M. However, the amino acid residues at position 145-147 (RGD) of VP1 were highly conserved in all virus isolates in Thailand including Lysine (L) at residue 148, which was important to antigenic properties of the virus. Besides, the amino acid position surrounding the RGD motif was also found to have several amino acid substitutions (Figure 12).

The N-terminal of RGD was found to have several amino acid substitutions. The amino acid residue at position 133 (N) was found to be conserved in all viruses isolated from Thailand but the substitution with S was found in O/189 and 2 viruses belonged to Cathay topotype isolated in 2005. Moreover, the O/189 strain had the alternative residues changing from consensus sequence at positions; 135 (K>R), 137 (A>S), 138 (E>N), 139(G>S), 140 (S>H), 141(L>V), 142(N>S) and 153(P>A). Amino acids at some positions were common for the certain groups such as arginine (R) at position135, serine (S) at position 139 and serine (S) at position 142 was commonly found in sublineage IID, lineage II and Pan Asia group, and 2 viruses of Cathay topotype, respectively.

The viruses isolated in Thailand during 1999 to 2001 belonged to the Pan Asia strain had the similar amino acid sequences (Figure 12). The residues 137 (G), 139 (S), 140 (P), 141(V) and 158 (T) were the common residues of Pan Asia strain from Thailand isolates with the exception for the 2 viruses differenced from O/189 that had the R substitution for Q at position 149. Two isolates belonged to Cathay topotype (O/F48/02/05 and O/F48/03/05) had several different residues from O/189 strain at position 134, 135, 137, 138, 139, 140, 141, 142, 156, 158 and 174. Moreover, these 2 isolates of Cathay topotype also had the deletion in 3A protein at amino acid position 93-102 and may be concerned for the infectivity of virus (Figure 13).

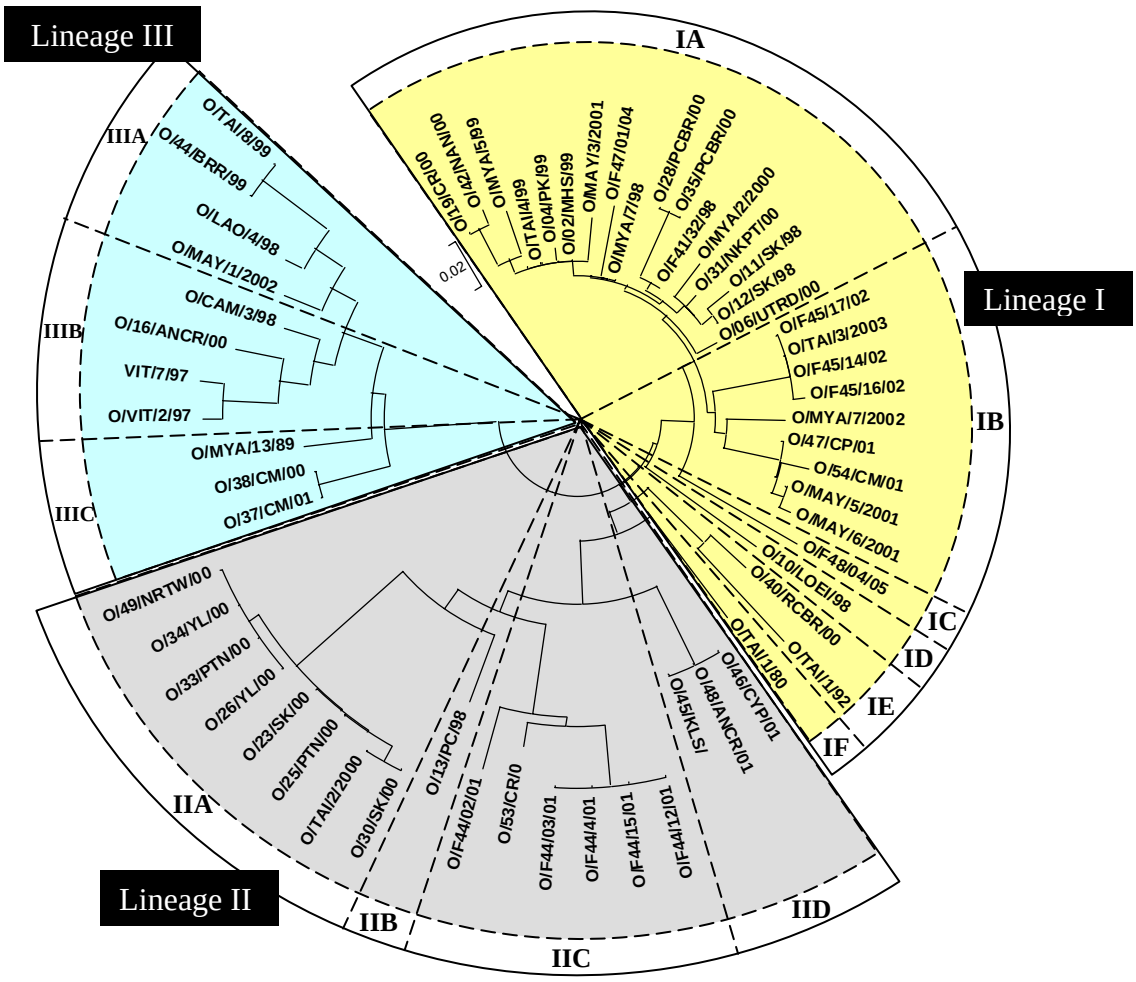



Figure 10 Phylogenetic tree of SEA topotype isolated in Thailand which was divided into 3 lineages (I, II and III) and sublineages of each lineage (A to F) based on nucleotide 361-550 of VP1. Dash lines separated sublineages.

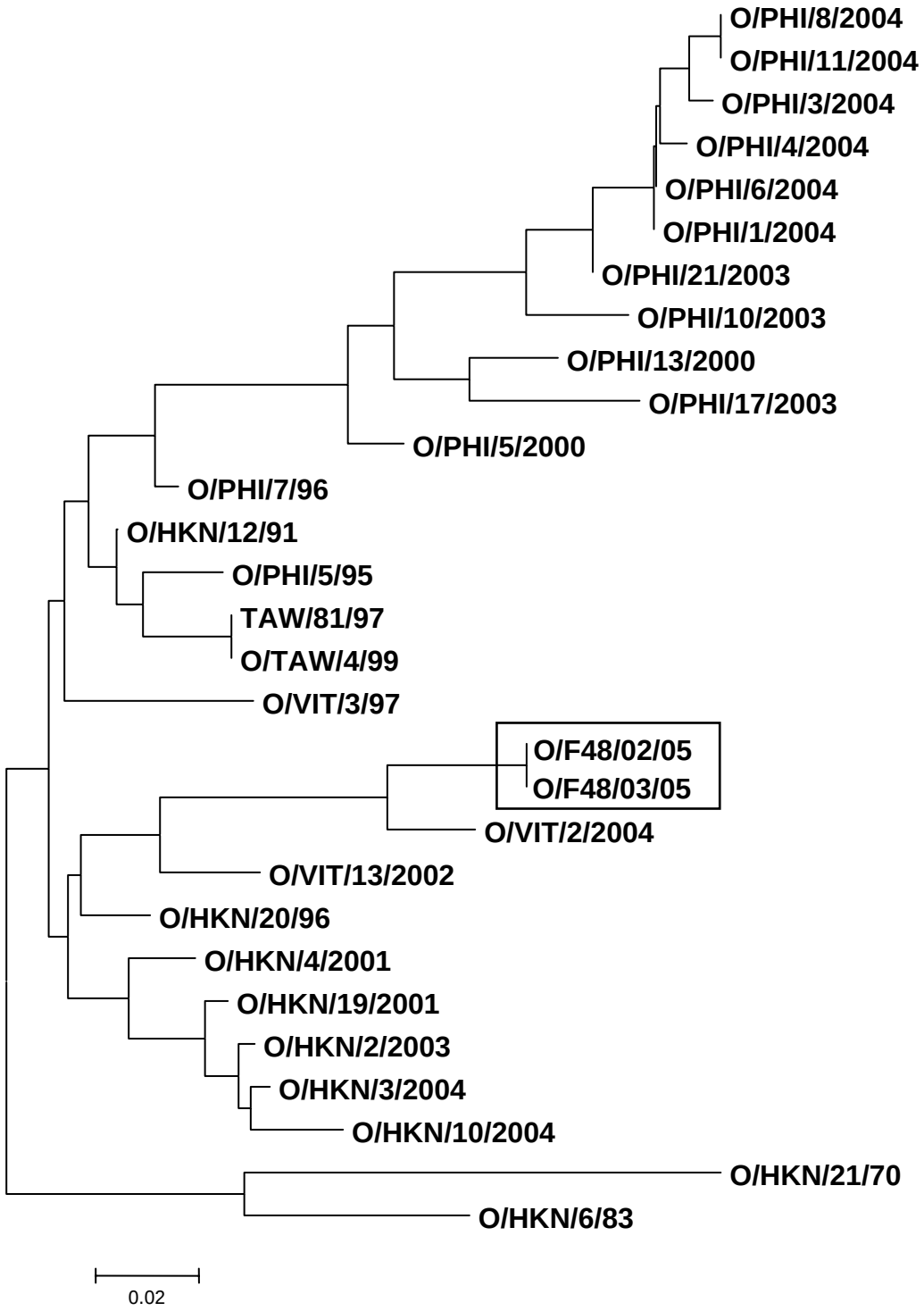



Figure 11 Phylogenetic tree of Cathay topotype. The isolates O/F48/02/05 and O/F48/03/05 (black box) were isolated from Thailand in 2005.

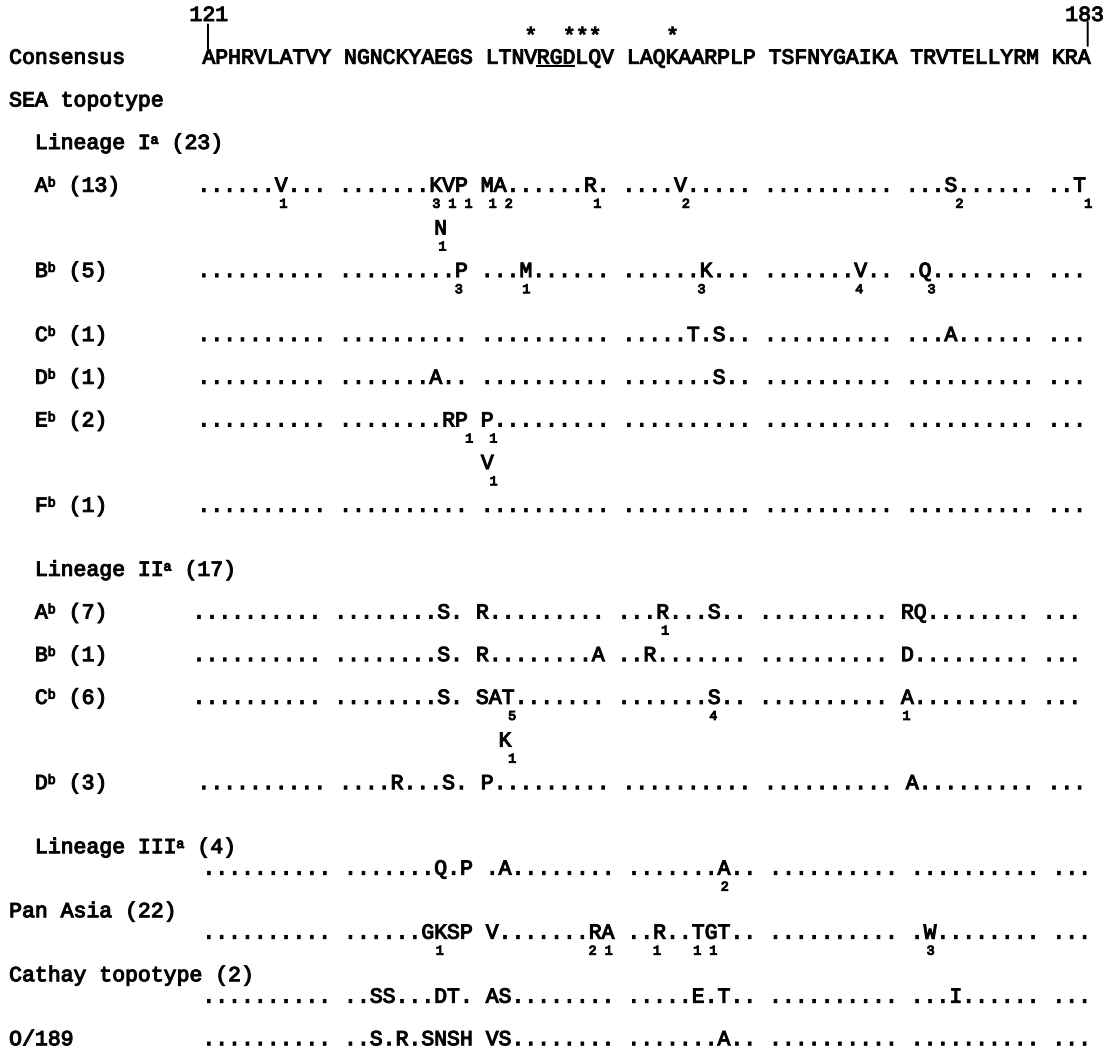



Figure 12 Amino acid residues of FMDV serotype O isolates in Thailand. The top row was consensus sequence. Underline was represents motif residues (RGD). Identical residues were shown as the dots (.). Asterisks were critical residues of the antigenic site. Total isolates were shown in the parenthesis. The numeric figure under the amino acid residue indicated the frequency of samples. The amino acid without the numeric figure showed conserved residue.

A = lineage of SEA topotype

b = sublineages

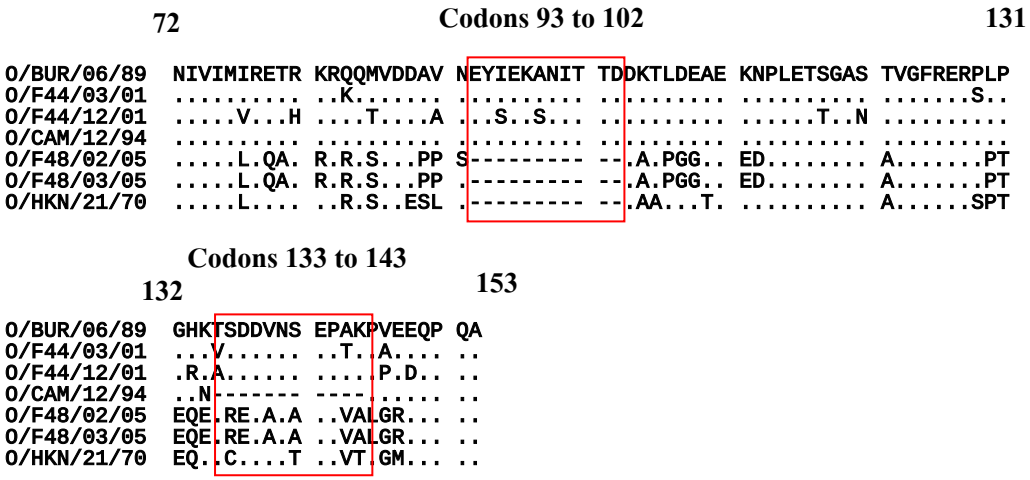



Figure 13 Comparison of amino acid sequences between positions 72 to 153 of the 3A coding region. Sources of the viruses isolated in Thailand were listed in table 3 and 5. Dots indicated identity with O/BUR/06/89 virus (accession; AJ294978) which displayed no deletion of 3A. The O/CAM/12/94 virus (accession; AJ294980) displayed the deletion of codons between positions 93 to 102. The O/HKN/21/70 virus (accession; AJ294984) displayed the deletion of codons between positions133 to 143. Dashes represented amino acid deletion.

**1.2 Serotype A**

The serotype A has been divided into 10 genotypes, designated as I-X, based on the complete nucleotide sequences of VP1 (Sanyal *et.al*., 2002; Tosh *et.al.,* 2002). The 49 nucleotide sequences of viruses isolated from Thailand during 1998 to 2003 including vaccine strains (A98/A22, A/100/SKNK/97 and A118/87) were calculated divergence percentage and used to construct dendrogram with the sequences obtained from GenBank for genotype classification. The viruses that circulated in Thailand were found to be separated from viruses from other countries by using dendrogram (Figure 14). The viruses were clustered into genotype IX as reported by Tosh *et al.* (2002). The maximum of divergence percentage was 10.3% excluding the vaccine strains. The divergence percentage of the isolates A98/A22, A/100/SKNK/97 and A118/87 comparative with the field isolates were 11.5-16.7%, 1.1-7.3% and 7.3-10.9 %, respectively. According to these results, all of the serotype A isolated in Thailand had only one lineage. However, the field virus strains can be classified into 3 sublineages (Figure 14). Sublineage A was the largest group of the viruses that circulated in every parts of Thailand during 1998 to 2002. Sublineage B contained 13 viruses isolated from the Northeastern part in 2001, and the Central and the Southern parts of Thailand during 2002-2003. The isolates A/80/KPS/01 and A/74/CYP/00 were in the sublineage C (Figure 15).

Critical amino acid residues of serotype A were 139,140, 141, 149, 153 and 173 which can observe amino acid substitution. The neutralization escape mutants due to the mutation in these critical amino acid residues have been identified (Tosh *et al.,* 2002). The majority of amino acid at residue 139 of field strains was proline (P) including A/100/SKNK/97, but only the A/66/PSNL/99 virus in sublineage A was leucine (L). The serine amino acid residue at position 139 was found in two vaccine strains (A98/A22 and A118/87). Both residues at position 140 and 141 were highly variation in amino acid residues. The majority of sublineage A was glycine (G) at residue 140. However, the virus isolated from the Central and the Northeastern parts of Thailand had glutamic acid (E) substituted for G at position 140. Moreover, the lysine (K) and valine (V) were found in one isolate out of 31 isolates and the V substitution was also found in vaccine strain (A98/A22 and A118/87). Most of the virus isolates belonged to

sublineage B had amino acid V at position 140, but the 3 isolates from the Northeastern of Thailand isolated in 2001 had G. The G also was investigated in sublineage C. The threonine (T) commonly found at residue 141. The minority of the isolates had either K or asparagine (N) or V or glutamine (Q) at residue 141. All of virus isolates had L at residue 149 but except A/100/SKNK/97 had phenylalanine (F). The majority of the virus isolates had isoleucine (I) at residue 153. However, the V at residue 153 was found in 2 isolates of sublineage A including 3 vaccine strains. The last critical amino acid residue, positioned173, was E but 5 viruses isolated in 2002 of sublineage B had Q substituted for E. Moreover, there was no amino acid change at RGD receptor (Figure 16).

### 1.3 Serotype Asia1

The viruses of serotype Asia 1 were classified into 6 lineages according to Sanyal *et al.* (2004). Two isolates (As/85/CR/01 and As/86/PSNL/01) belonged to lineage II of serotype Asia1 were isolated from Chiang Rai and Phitsanulok in 2001 (Figure 17). These viruses had 2.1% of divergence percentage from each other, but was found to have 0.5 and 3.2% with viruses isolated in 1998 (Asia1/TAI/1/98), respectively. Amino acid residues of viruses isolated in Thailand were not found substitution among these 2 isolates in this study and one isolate obtained from GenBank (Asia1/TAI/1/98) (Figure 18).

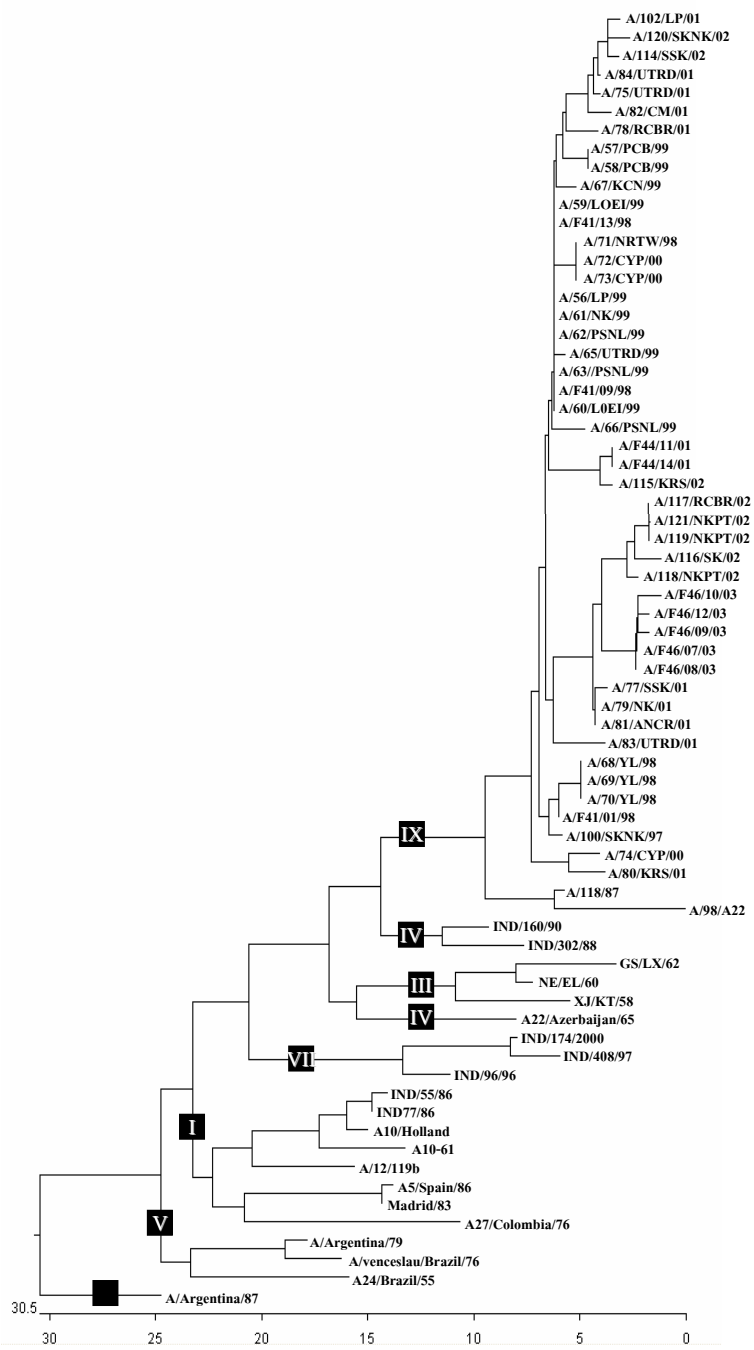



Figure 14 Phylogenetic tree among serotype A of viruses isolated in Thailand and those obtained from GenBank based on nucleotide 361-550. Genotypes were designated as I-X.

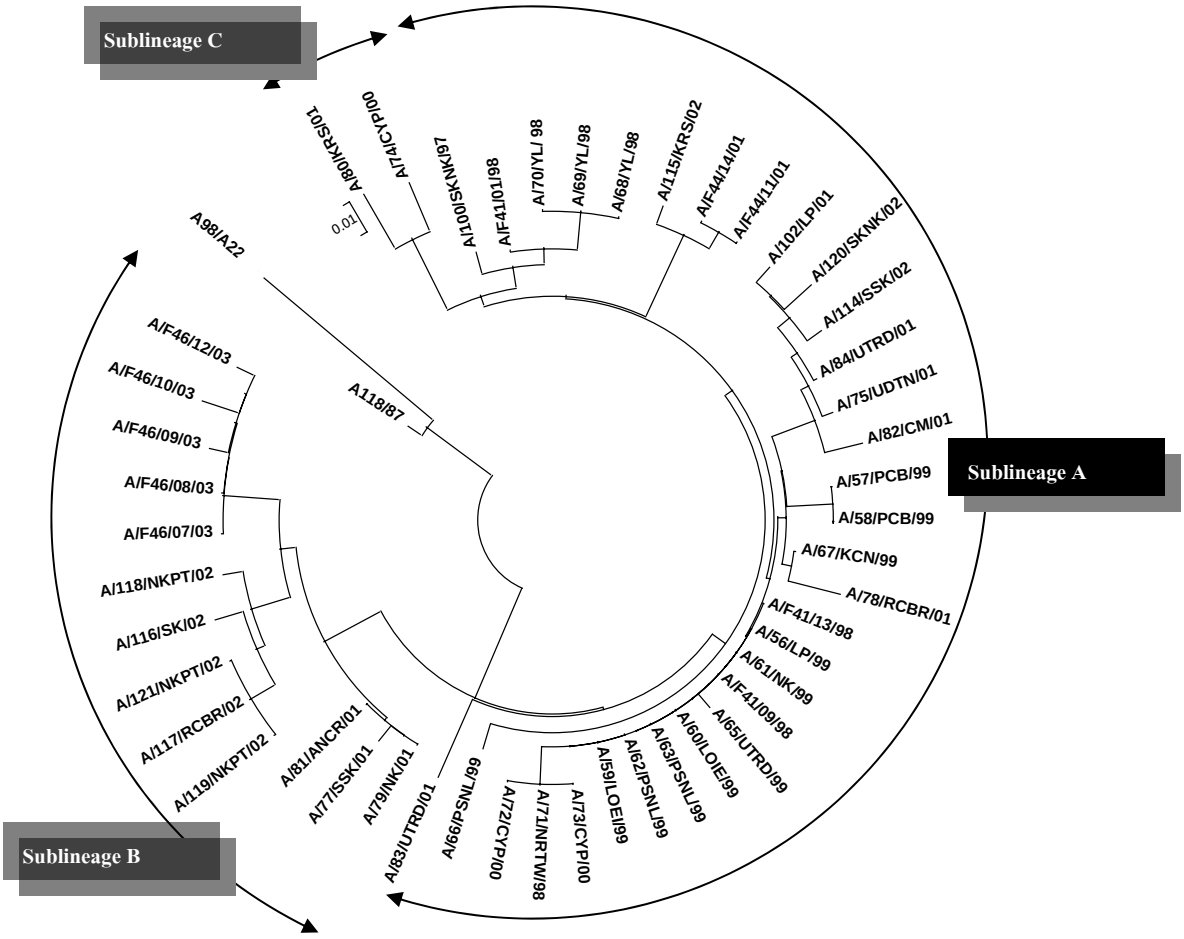



Figure 15 Phylogenetic tree of serotype A viruses isolated in Thailand based on nucleotide 361-550 of VP1. They can be divided into 3 sublineages (A-C). The black arrows represented sublineage areas. The A/98/A22, A/100/SKNK/97 and A118/87 were vaccine strains.

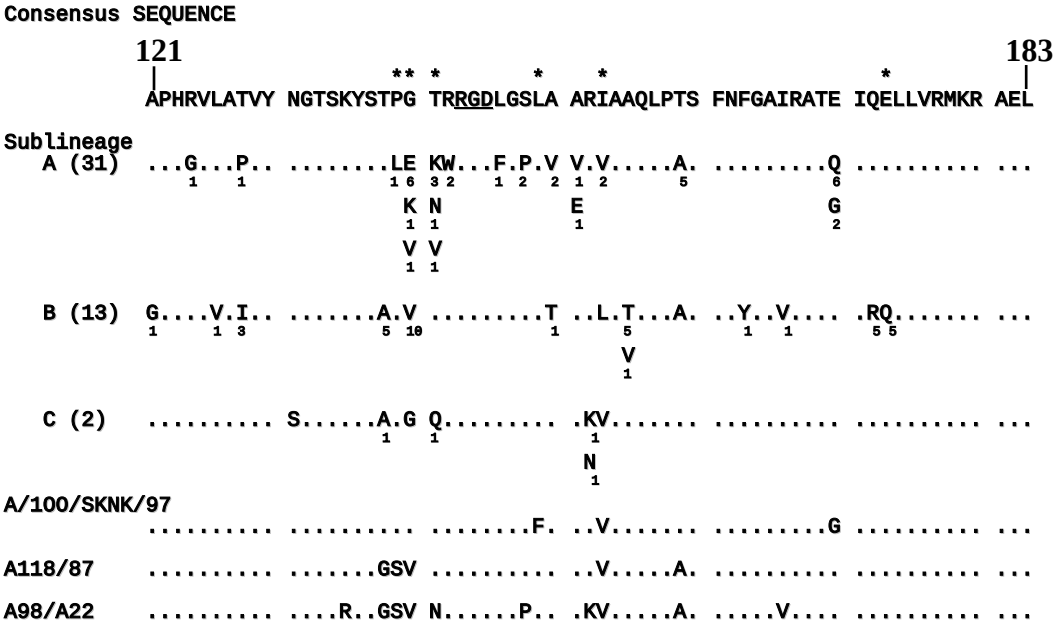



Figure 16 Amino acid residues comparisons of serotype A were aligned. The consensus sequence was drawn on top line. The underline represented motif residues (RGD). The state of identical residues was shown as a dot (.). Asterisks were critical residues of the antigenic site. Total isolates were showed in the parenthesis. The numeric figure under the amino acid residue indicated the frequency of samples. The amino acid without the numeric figure showed as the conserved residue.

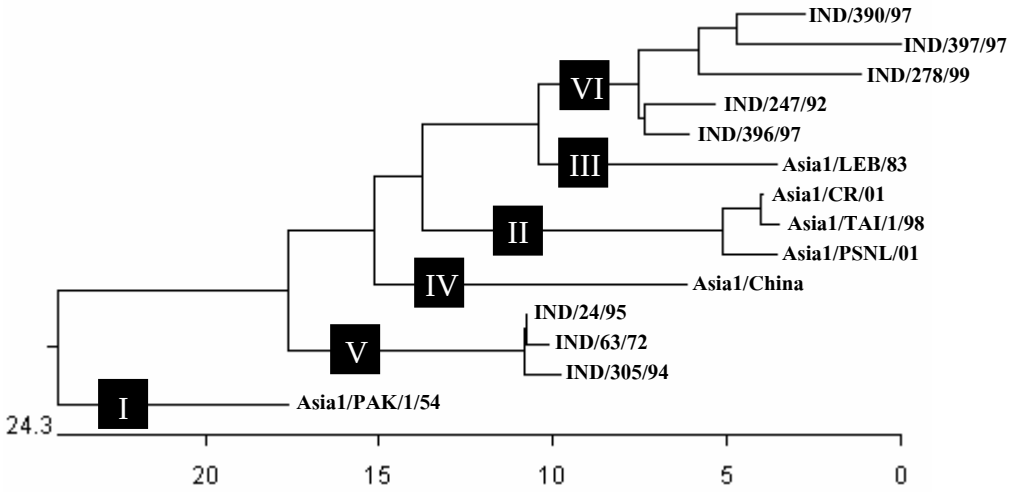



Figure 17 Phylogenetic tree and lineages of serotype Asia1 based on nucleotide 361-550 of VP1. Genotypes were designated as I-VI.


Figure 18 Amino acid residues of serotype Asia1 at the position 121 and 183 showed conserved in 2 virus isolates from this study and including one isolate obtained from GenBank database. The underline represented motif residues (RGD).

**2. Synthetic Peptide Vaccine**

Pigs immunized with synthetic peptide vaccine (group 1) had SN titer against O/189 (reference strain) higher and faster than pigs immunized with commercial vaccine (group 2) (Table 9). For group 1, high SN titer was initially found in 2 weeks after 2nd vaccination and prolonged up to 4 weeks. For group 2, Three out of four pigs gave SN titer less than 1.0 since first immunization until 4 weeks after 2nd vaccination excluding one pig gave value of more than 1.0 after 2 weeks of 2nd vaccination. The control group had no response according to both SN test and UBI® FMDV VP1 EIA test. Moreover, antibody response against VP1 evaluated by UBI® FMDV VP1 EIA test based on VP1 synthesis peptide had the results correlated with SN test (Table 9). After challenged with $10^7$ $TCID_{50}$ of field strain virus, SN titers to serotype O/189 of group1 and group2 were 1.8 to 2.64 and 1.2 to 1.68, respectively. The UBI® FMDV VP1 EIA test showed positive results. The control group did not response to both SN and UBI® FMDV VP1 EIA tests. Even though all groups of pigs showed no lesions. Sera from these pigs were tested for viral replication by UBI® FMDV NS EIA test kit. All of pigs gave negative results with an exception for one pig from group 1 gave the positive result (0.345). Moreover, two pigs from group1 had SN titer against serotype Asia1, and three pigs from group 2 also had the response to both serotype A (A/Sakon Nakhon/97) and Asia1 (Table 10).

The sequence of amino acid residues of synthetic peptide vaccine were used to compare with the sequence of amino acid residues of virus isolates from Thailand (Figure 19). The critical residues of serotype O at positions 144, 147, 148, 149 and 154 of synthetic peptide vaccine and virus isolates from Thailand were identical. However, the residues at the N-terminal part of RGD motif showed highly variation.