EXPRESSION OF RECOMBINANT VP1 PROTEIN OF FOOT AND MOUTH DISEASE VIRUS SEROTYPE O IN *ESCHERICHIA COLI*

INTRODUCTION

Foot-and-mouth disease (FMD) is the economically most important animal viral disease world wide (Domingo *et al*., 2002). It classified as a List A disease by "Office International des Epizooties (OIE; World Organization for Animal Health) (OIE, 2004). The List A diseases have the potential for rapid and extensive spread within and between countries. (Callens and Clercq, 1997; Davies, 2002; Alexandersen *et al*., 2003). The effected countries cannot participate in international trade of animals and animal products (Domingo *et al*., 2002). As illustrated by the epidemic in the UK in 2001. It has been estimated that the direct cost of epidemic was 2.75 billion ponds. The indirect costs from the combined losses of agricultural exports and tourist trade are difficult to accurately estimate (Alexandersen *et al*., 2003). FMD is endemic in large areas of Africa, Asia and South America (Blood and Radostits, 1989). Moreover, it has ability to cross international boundaries and causes epidemics in previously free areas including Europe (Knowles and Samual, 2003). Currently, the high capacity of this disease spread is probably favorable by the growing globalization of trade, which facilitates dissemination of the disease (Saiz *et al.*, 2002).

Foot-and-mouth disease (FMD) is a viral infectious disease of cloven-hoofed animals. This disease is caused by foot-and-mouth disease virus (FMDV). The prominent clinical signs are fever, vesicles in mouth and feet (Blood and Radolits, 1989). Although the mortality rate is very low, it is fatal in young animals. Surviving cattle are subsequently carry virus for up to two years (Blood and Radolits, 1989). These animals will be reservoir of a following outbreak (Saiz *et al.*, 2002).

There are several approaches to control FMDV. Effective approaches are vaccination and slaughter (Blood and Radolits, 1989; Davies, 2002; Domingo *et al*.,

2002). The vaccines currently available are inactivated vaccines (Saiz *et al*., 2002; Doel, 2003). Although it is an effective tool to prevent, the risk in the dissemination of virus from the factories and the problem of vaccine transport and maintenance are concerned (Carrillo *et al*., 1998). As a result, it is important to develop alternative vaccines that lack of a complete FMD virus. Peptide, DNA and edible vaccines are the novel types of vaccine. They have been research extensively for the recent year. The new vaccines have advantages over the conventional vaccines by reducing risk of outbreak from uncompleted inactivated virus or escape viruses from vaccine factories.

FMDV capsid proteins have been study for subunit vaccines. Among those, VP1 protein is prevalent. VP1 protein, the major capsid protein of FMDV, carries neutralizing epitope including B and T cell epitope. Neutralizing epitope responses to the induction of protective neutralizing antibodies (Brown *et al*., 2000). The rVP1 expressed ability to stimulate immune response against FMDV and protected immunized animals from homologous FMDV challenge (Grubman, *et al.,* 1993; Wang *et al.*, 2003; Shi *et al*., 2006). VP1 protein has been successfully expressed in various systems, such as in transgenic plants (Carrillo *et al*., 1998; Wigdorovitz *et al*., 1999a, 1999b; Cedillo *et al*., 2001; Santos *et al*., 2002), *Pichia pastoris* (Shi *et al*., 2006), insect cell (SF9) (Grubman, *et al.,* 1993), and in *E. coli* (Kleid *et al.*, 1981; Wang *et al.*, 2003).

In present study, recombinant VP1 protein was expressed in *E. coli*. The rVP1 was used as antigen to immunized rabbits. The antibody against FMDV was determined by ELISA and serum neutralization techniques. It had a potential to stimulate protective immune response in tested rabbits.

OBJECTIVES

1. To clone and express VP1 protein of FMDV serotype O, Thai isolate in *Escherichia coli*

2. To test the ability of the recombinant VP1 protein to induce immune response in rabbits

LITERATURE REVIEW

1. Food and Mouth Disease (FMD)

FMD is the first animal disease that is attributed to a virus. The causative agent is foot-and-mouth disease virus (FMDV) (Doel, 2003). It is a severe, clinically acute, vesicular disease of domestic cloven-hoofed animals, such as cattle, sheep, goats, llamas, camel, and swine and more than 70 species of wild cloven-hoofed animals (Diego *et al*., 1997; Murphy *et al*., 1999; Alexandersen *et al*., 2003). The American buffalo (*Syncercus caffer*) appears to be susceptible to infection and may act as a reservoir host. Although FMD is known as a disease of cloven-hooped anials it can occure naturally in other animals, for example, the hedgehog (*Erinaceus spp*.) (Davies, 2002). FMD affects extensively areas of the world (Saiz *et al.,* 2002).

Figure 1 Countries in which FMD was reported (■) in 2005-2006.

Source: http://www.biwa.ne.jp/~sigakaho/tsushin/67/FMD.ht1.jpg

1.1 Pathogenesis

Route of transmission

FMD can be transmitted by both directed and in directed mechanisms. The most common mechanism of transmission of FMDV is a direct contact by mechanical transfer of virus from infected to susceptible animals. It may be facilitated by physical contract with infected excretion or secretion, including vesicle fluid, milk and epithelium containing FMDV. The virus enters hosts through cuts or abrasions skin or through the mucosa of respiratory and oral routes. Normally, the intact epidermis provides protection, except traumatic or infection. Livestock with damage to the integument are highly indirectly infected by contaminated personnel, vehicles, and all classes of fomites. FMDV can also causes infection in the respiratory tract of recipient animals by virus in aerosol or infected debris. This is the main rout of FMD infection (Saiz, 2002). Among FMDV host, pigs are relatively resistant to aerosol exposure. FMD can also be transmitted via oral route. For example, the South Africa 2000 and UK 2001 epidemics have been attributed to the feeding of unheated waste food to swine (Davies, 2002; Alexandersen *et al*., 2003).

Incubation period

The incubation period of FMD is highly variable, and depends on the strain and dose of virus, the route of transmission, the animal species and the husbandry conditions. The incubation period for farm-to-farm airborne spread ranges from 4 to 14 days by indirect contact and 2 to 14 days by direct contact. For within-farm spread, the period is generally 2–14 days but may be as short as 24 hours. Under field conditions, the dose of FMDV will be influenced by many factors, especially the stocking density and housed ventilation. Some activities, for example, animal examination, vaccination, and marketing will accelerate the rate of spread of virus in an infected flock or herd (Saiz *et al*., 2002; Alexandersen *et al*., 2003).

Sites of viral infection and replication

For both direct and indirect air born infection, pharynx is a primary target of FMDV (Saiz *et al*., 2002). This organ favors for infection due to stratified squamous epithelium (a living cell type) lines on the surface. In contrast, the other organs in oral cavity are over lay with stratified squamous and cornified cells (dead cells). Organs covered by a layer of dead cells are much more likely to be infected from viraemia. In sheep, tonsil plays a crucial role in initial infection because it located immediately above the dorsal soft palate (Davies, 2002; Alexandersen *et al*., 2003).

Viral replication takes 2-3 days after exposure at sites of infection. Less of viral replication occurs in the epithelia of the skin and mouth during the acute phase of the disease. Interestingly, nasal and lung are not sites of viral replication. Replicated virus reaches its peak in 2-3 days. The virus then transfer to regional lymph nodes before spread through blood stream, called viraemia, and then to cornified stratified squamous epithelia of the skin, feet, mammary gland, mouth, and tongue, or the myocardium of young animals. Viraemia usually lasts for 4–5 days.

Clinical signs and symptoms

The severity of clinical signs varies with the strain of virus, the exposure dose, the host species, the animal age, breed of animal, and its degree of immunity. This disease is severe in swine and cattle, but in some species the infection may usually be sub-clinical. For example, African buffalo (*Syncerus caffer*) (Murphy *et al*., 1999; OIE, 2004).

The characteristic of foot-and-mouth disease is an acute febrile reaction and the formation of vesicles in and around the mouth and on the feet. The resultant pain causes lameness, manifested by foot “flicking” and reluctance to stand or walk. Feet lesions are often observed initially as blanched areas, which

subsequently develop to vesicles. Vesicles on the feet of pigs and cattle are most often seen in the interdigital space (at the bulb of the heel) and along the coronary band. Vesicles may also be seen on the snout or muzzle, teats, mammary gland, vulva and other sites of the skin. The most consistently vesicle, however, is in and around the mouth and on the feet.

Lesions in the mouth of large and small ruminants are most often seen on the dental pad and the tongue but may also be seen on the lips, gums, and cheeks, and sometimes on the hard palate. In pigs, mouth lesions are most often located on the tongue. FMD in small ruminants may be clinically inapparent (Donaldson and Sellers, 2000). As in sheep and goats, oral lesions in pigs may heal without much exudate or subsequent scarring. Soon after rupture, the base of vesiculated areas becomes covered within a few days by a serofibrinous exudate. The regeneration of epithelia is usually well within 2 weeks. Secondary infections may complicate and prolong the healing processes.

More than lesion, FMD also causes abortion in pregnant animals. Death may result in some cases. Mortality from a multifocal myocarditis is most commonly seen in young animals. Moreover, myositis may also occur in other sites (Alexandersen *et al*., 2003).

Viral excretion

Virus is excreted from infected animal by all means of excretion. Saliva, nasal and lachrymal fluid, milk and expired breath may contain virus during the prodromal period. Urine and faeces also contain virus but only small amounts. Viral excreted from infected animals varies between animal species. Studies in sheep demonstrated that virus was detectable in the breath 1-2 days before clinical signs. By contrast, the peak of airborne viral excretion in cattle and pigs occurred during the viraemic phase. Among domesticated animals, pig liberates the largest quantities of airborne virus than either cattle or sheep. Ruminants excrete less of virus in their

breath but, in contrast to pigs, are highly susceptible to infection by the respiratory route (Sellers *et al*., 1969).

A sharp decline in viral excretion occurs around day 4–5 of clinical disease when antibodies are produced. Animals are free of detectable infectivity at 10–14 days post-infection. However, virus already excreted during the preclinical and acute clinical phases.

Antibody response against FMDV

Infected and vaccinated animals produce antibodies against FMDV. The virus stimulates both B and T cell rapid humeral specific response in infected animals (Zamorano *et al*., 1995). The neutralizing antibodies are directed to B-cell epitope located on the viral capsid. A high level of antibodies can be detected soon after infection or vaccination (Saiz *et al*., 2002). Specific T-cell response is also elicited. In cattle and swine, B-cell activation and antibodies production are associated with a lymphoproliferative response mainly to T helper cells. They recognize number of epitopes located both in capsid and non-structural proteins. FMDV infection results in rapid reduction of MHC class I expression on the surface of susceptible cells. This effect could impair the presentation of virus epitope by FMDV infected cells to CTL response of the host (Saiz *et al*., 2002). Interferons (alpha, beta, gamma) also play roles in FMD, as well as certain interleukins and perhaps tumour necrosis factor alpha (TNF-α). Such development might even lead to a cure for persistant infection and contribute to the ultimate epidemiological challenge the elimination of FMD (Alexandersen *et al*., 2003).

Circulating antibodies to FMDV can be detected from 3 to 4 days after the first clinical signs. They are relatively efficient in clearing virus from the circulation. Clearance of virus is correlated with the concentration of circulating antibodies. The clearance is less efficient at peripheral sites. Therefore, virus may

remain in vesicular epithelium for up to 10–14 days and detectable for longer periods in foot lesions than in mouth lesions (Oliver *et al*., 1988).

Unfortunately, recovery from infection, or protective vaccination, with one serotype will not protect against subsequent infection with anothers. Moreover, within a serotype a wide range of strains may occur. Some divergent may be sufficiently reduce the efficacy of existing vaccines (Kitching *et al*., 1989, 1998; Alexandersen *et al*., 2003).

Carrier state

After recovery from illness, animal may become a persistent infection (a carrier stage). A persistent infection defined as the presence of detectable virus for at least 28 days after infection (Davies, 2002). In persistent stage, the target region for infection in cattle is the pharynx. However, the mammary gland and testicles may contain virus for several weeks after infection. Pituitary grand, pancreas and thyroid also have been suggested as additional sites of persistence. Organs such as the kidneys and lymph nodes are not likely to be sites of persistence. However, pharyngeal sites have the ability to support viral replication in the presence of circulating antibody. The carrier virus is fully infectious and consequently the carrier state is associated with a theoretical risk of spreading the disease (Mezencio *et al*., 1999; Davies, 2002; Alexandersen *et al*., 2003).

Persistent infection can be found in a proportion of infected cattle, sheep and goats. Pigs, however, cleared FMDV in 3-4 weeks and did not become carriers. The maximum reported duration of the carrier state in different species is 3.5 years in cattle, 9 months in sheep, 4 months in goat, 5 years in African buffalo, and 2 months in water buffalo (Davies, 2002; Zhang and Alexandersen. 2003).

1.2 Diagnosis

The clinical diagnosis of FMD is sometimes difficult, especialy in sheep and goats, in which clinical signs are often mild (Callens *et al*., 1997; Barnett and Cox, 1999; Donaldson and Sellers, 2000; Alexandersan *et al*., 2002; Hughes *et al*., 2002). Moreover, some strains of the virus may be low virulence for some host species. In addition, several other viral vesicular diseases, including swine vesicular disease, vesicular stomatitis and vesivirus infection, cannot be distinguished from FMD only on the basis of clinical findings. Thus, a definitive diagnosis requires laboratory investigation. (Alexandersen *et al*., 2003; OIE, 2004). Diagnosis of FMD is by detection of FMD viral antigen, its nucleic acid and a specific humeral antibody (Saiz *et al.,* 2002; OIE, 2004).

There are several methods to detect the viral antigen. First is viral isolation. This method requires viral amplification from collected sample in cell culture. The isolated virus is then used to infect cell line and record for generated cytopathic effect (CPE). This procedure takes time and lab safety. The second method is complement fixation (CF) test. It has been a traditional test for diagnosis. Currently, CF has been replaced by the ELISAs. ELISA technique has largely been used due to their sensitivity, specificity and ability to deal with large number of samples. However, CF can be performed if ELISA is not available. Instead of detect antigen, FMDV can be diagnosed by detection of its genome presented in samples. By using specific primers, FMDV could be distinguished between each of the seven serotypes (Remond *et al*., 2002). Additionally, *In situ* hybridization techniques have been developed for investigating the presence of FMD virus RNA in tissue samples. This technique is only in use in specialized laboratories (OIE, 2004).

FMD can be diagnosed by a detection of a specific antibody response to structural proteins. The generally used tests are virus neutralization (VN) and ELISA. Low titer false-positive reactions can be expected in a small proportion of the sera in either test. Screening by ELISA and confirming the positives by the VN test minimize the occurrence of false-positive results (Remond *et al*., 2002; OIE, 2004).

The infected animals can be discriminated from vaccinated by non-structural proteins which can be detected only in infected animals. There are several specific ELISA to detect non-structural proteins (Sorensen *et al*., 1998). For example, a MAb trapping (MAT) ELISA for detecting antibody to 3ABC and blocking ELISAs for detecting antibody to 3AB or 3ABC. Currently, the Enzyme-linked immuno-electrotransfer blot assay (EITB) has been widely applied in South America for serosurveillance and risk assessment associated with animal movement. Another interesting method is a test strips containing the purified recombinant antigens of NS proteins (3A, 3B, 2C, 3D and 3ABC). These proteins are expressed in *E. coli* C600. A sample is positive if all four antigens (3ABC, 3A, 3B and 3D) reactive at equal to or higher than the cut-off value (OIE, 2004).

1.3 Viral survival in environment

Few data are available on the persistence of FMDV in the environment (Sanson, 1994; Cottral, *et al.,* 1969). Most FMDV strains are stable within the pH range 7.0–8.5, especially at lower temperatures. Labile is increasing at pH values outside that range. Excreted virus can survive in the environment for weeks or even months (Davies, 2002). In general, the environmental factors that favor survival of virus are moist conditions, neutral pH and low temperature. Survival of the virus in aerosols depends on the relative humidity (Donalson *et al*., 2001; Davies, 2002). However, air born spread is a feature of the disease in moist temperate as opposed by dry tropical climates (Davies, 2002). The viral survival also depends on the nature of the contaminated material. There have been reports of survival of the virus for 200 days on hay, for 6 months in faecal slurry in winter (Hyslop, 1970), 4 weeks on cow's hair at 18–20^{o}C, 14 days in dry faeces, 39 days in urine, 3 days on soil in summer, and up to 28 days in autumn. In meat, the acidity produced in carcass meat during rigor mortis in cattle will inactivate the virus. In contrast, the pH in bone marrow, lymph nodes and certain organs does not decline during rigor mortis. Virus can, therefore, be found in such material for an extended period of time, and may cause new outbreaks if fed to livestock as unheated waste food (Alexandersen *et al*., 2003).

1.4 Epidemiology

The epidemiology of FMD is complex because all secretions and excretions from infected animals become infectious. Such fluids are important in viral spread. (Alexandersen *et al*., 2003). In addition, viruses can survive in environment for a period of time. There are several common methods of spread of FMD. The most important method includes movement of infected animals, contaminated frozen meat. Direct contact between the infected and susceptible animals, the feeding of contaminated animal products to susceptible livestock. Indirect contact, for example, contact with virus from infected animals transported mechanically by persons and livestock vehicles also leads to spread of virus (Davies, 2002; Alexsandersen *et al*., 2003). The environment can provide geographical barriers to virus dissemination or, alternatively, can promote virus transmission when appropriate atmosphereic condition prevail (Saiz *et al*., 2002). Wind may spread the virus over long distances as far as 100 km and possibly across expanses of sea (Blood and Radostits, 1989; Diego *et al.*, 1997; Murphy *et al*., 1999). For example, it was discovered that airborn virus spread could infect animals some distance away I FMD epidemic in the UK in 1967 (Davies. 2002).

2. Food and mouth disease virus (FMDV)

FMDV is belonged to family *Picornaviridae*, genus *Aphthovirus*. There are seven serotypes (O, A, C, Asia1, SAT1, SAT2 and SAT3) and numerous subtypes are reported through the world (Murphy *et al*., 1999; Woolhouse *et al*., 2001). They do not cross-protection in infected animals (Davies, 2002). Foot-and-mouth disease virus (FMDV) is a small, non-enveloped single stranded, positive sense RNA virus. The genome is encapsidated in an approximately 26 nm in diameter icosahedral shape capsid. The capsid composed of 60 copies of four proteins (VP1, VP2, VP3, and VP4) (Jackson *et al*., 1997; Alexanderson, 2003).

2.1 FMD genome

The molecular epidemiology of FMDV has progressed in the last 15 years. The viruses are able to divide into genotypes based on 15% nucleotide differences of VP1 sequence. In case of the SAT serotypes, it was raised to 20% since the VP1-coding sequence of these viruses appears to be more inherently variable (Samuel and Knowles, 2001).

The seven serotypes of FMDV are not distributed equally around the world. The SAT serotypes are normally restricted to sub-Saharan Africa. Type O and A have the broadest distribution occur in many parts of Africa, Southern Asia, the Far East (not type A) and South America. Type C appears to have become confined to the Indian sub-continent. Serotype Asia 1 normally only occurs in southern Asia. Currently, Europe, North and Central America, Greenland, Australasia and Oceania are FMD-free (Knowles and Samuel, 2003).

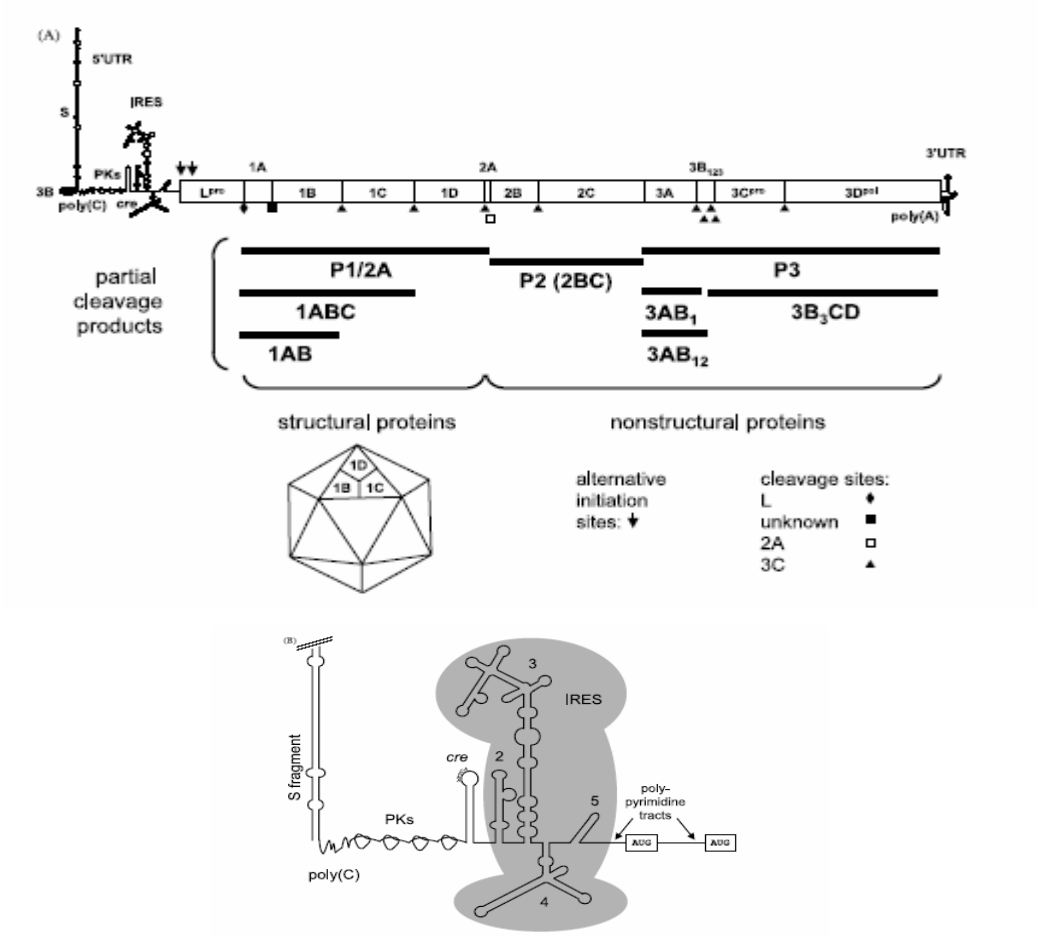

Figure 2 Schematic diagram of the FMDV genome

(A) Overview; open boxes indicate protein-encoding regions, lines indicate RNA structures (1A=VP4, 1B=vp2, 1C=Vp3, and 1D=VP1). Thick lines below the genome diagram indicate prominent partial cleavage products. (B) Expanded view of the 5'UTR; S fragment, short fragment of the genome, poly(C), poly cytosine tract, PK, pseudoknot, cre, cis-acting replicative element, IRES, internal ribosome entry site.

Source: Mason *et al*. 2003.

The genome of FMDV is 8-8.5 kilobases in length and contains a single long open reading frame (ORF). It can be divided into three main functional regions: 1) the 5' untranslated region (UTR), 2) the protein coding-region (L/P1, P2 and P3) and 3) the 3' untranslated region (Figure 2).

These UTRs that surround the coding region involve in viral virulence, stability and also provides initiation of viral translation including viral replication. At 5' UTR, FMDV genomes do not contain the 5'-terminal 7-methyl-G cap structures but with pUp. The pUp is the virion-associated genomes that covalently linked to 3B protein. The following genome segment from 5' UTR are S-fragment, poly(C), pseudoknot (PKs), *cre*, and an internal ribosome entry site (IRES). These structures involve in RNA replication and initiatiation of genes translation. IRES locates upstream to the initiating AUG codon in the L gene. IRES can directly interact with host eIF4G, and then shut-off host cell protein synthesis. The cleaved portion of host eIF4G and IRES complex binds with the small ribosomal subunit to initiate viral protein translation. Additionally, IRES should play a role in viral pathogenicity (Domingo *et al*., 2002; Saiz *et al*., 2002; Mason *et al*., 2003).

At 3' UTR of the picornavirus genome contains a poly A. Unlike cellular mRNAs, the poly (A) tract of picornaviruses is encoded in the genome. It plays a role in RNA replication and viral stability. A deletion of most of the 3'UTR region resulted in a total inhibition of viral replication.

The coding region of FMDV genome contains genes that encode for over a dozen well-described non-structural and structural proteins.

Non-structural protein coding region

The non-structural proteins (NSPs) of Picornavirus encoded from genes in L gene, P2 and P3 part. NSPs participate in RNA replication, virulence, structural protein folding and assembly. The first gene is Lpro gene codes for leader proteinase (Lpro). It is important determinance of FMDV virulence. Lpro protein responses for shut-off of host protein synthesis by degradation of host translating initiation factor (eIF4G) and freely uses the host protein synthesis machineries for viral protein synthesis. In this region, there are two in-frame AUG codons that encode proteins termed Lab and Lb. The Lb protein which is synthesized from the second AUG

codon is the major protein synthesized *in vivo* (Domingo *et al*., 2002; Saiz *et al*., 2002; Mason *et al*., 2003).

The P2 region of the picornavirus can be processed into three mature polypeptides, 2A, 2B, and 2C. The 2A peptide remains associated with the P1 structural protein precursor. It appears to be an autoproteinase. The 2B and 2C proteins have been implicated in virus-induced cytopathic effects (CPE). The 2B protein has been shown to enhance membrane permeability and block host protein secretory pathway.

The P3 region consists 3A, 3B, 3C, and 3D protein. FMDV 3A and 3AB play a critical role as an RNA replication factors. FMDV can be differentiated from other picornaviruses by three copies of 3B. The 3B protein contains the Tyr that can phosphodiester links to the viral RNA (Forss and Schaller, 1982). The 3Cpro protein of FMDV is identified as a proteinase. It is responsible for most cleavages in the FMDV polyprotein. The 3Cpro protein also cleaves host eIF4A. Moreover, it functions as an RNA helicase. In addition, the 3CD protein participates in formation of ribonucleoprotein complexes to influence both replication and translation. The FMDV 3D protein is the viral RNA polymerase. The 3Dpol nucleotides are highly conserved among the different FMDV sero- and subtypes (Domingo *et al*., 2002; Saiz *et al*., 2002; Mason *et al*., 2003).

Structural protein coding region

The P1 region encodes for capsid proteins. It is composed of 60 copies each of the four structural proteins, 1A, 1B, 1C, and 1D. They are also called VP4, VP2, VP3, and VP1respectively. The VP1 –VP3 line exterior while the VP4 buries within the virion. Therefore, the entire capsid surface is covered by portions of the other three proteins (VP1-VP3). The cores proteins VP2, VP3, and VP1 form a highly conserved eight-stranded β-barrel (A-H loop) of viral capsomer (Domingo *et al*., 2002; Saiz *et al*., 2002; Mason *et al*., 2003).

FMDV life cycle

FMDV can infect host via parenteral, oral, and nasal routes. The mechanism that the virions initiate infection is based on the attachment of the RGD motif to host receptor (integrins) (Pierschbacher and Ruoslahti, 1984; McKenna *et al*., 1995; Rieder *et al*., 1996; Alexanderson, 2003; Jackson *et al*., 2003). The RGD tripet is located on the surface G-H loop of VP1 capsid protein. Unlike other picornaviruses, FMDV is susceptible to low pH-induced disassembly. Therefore, the acidity environment in cytoplasm dissociates the viral capsid (Mason *et al*., 2003) and releases the viral genome (Davies, 2002). The site of RNA genome replication is a membrane-bound replication complex at the cell cytoplasm (Domingo, 2002). In all picornavirus infections, the first step in RNA replication consists of synthesis of a minus-strand RNA molecule which is incorporated into a replicative form where positive-strand progeny genomes are produced. There is little known about RNA replication in FMDV infected cells. As cytoplasmic replication occurs, viral RNA is translated (Davies, 2002).

Since FMDV contains only one open reading frame, the first translated protein is a polyprotein precursor. Subsequently, the P1/2A precursor is processed (Figure 1). The precise nature of the cleavage mechanisms utilized by 2A and the maturation cleavage of capsid protein 1AB into1A and 1B remains unclear. Accomplished by 3Cpro, a precursor protein transforms to a protomer that contains one copy each of 1AB (VP4 and VP2), 1C (VP3), and 1D (VP1). Five protomers assemble into a pentamer and twelve pentamers can assemble into an RNA-containing particle, called a provirion (Figure 3). The complete viral capsid consists of 60 copies of each of the four structural proteins, VP1-VP4. Assembly of the viral capsid and encapsidation of the viral RNA occur by mechanisms which are still obscure. In the final step, the viruses released from infected cell by cell lysis and the amplified viruses enter the neighboring cells.

3. Foot-and-mouth disease control

FMDV has a wide host range, an ability to infect in small doses, a rapid rate of replication, a high level of viral excretion and multiple modes of transmission. These features make FMD a difficult and expensive cost to control and eradicate.

The objective in eradication and control is to stop transmission by reducing virus emitted from infected animals and contact animals as quickly as possible. The methods applied are based on knowledge of the epidemiology of the disease. FMD control requires the earliest identification, movement restrictions and disinfection. Cleansing and disinfection is effective in eradicating the agent from infected premises (Davies, 2002). Vaccination has been applied in endemic area to prevent infection. In case of FMD out break, animals are vaccinated to enclose the outbreak before slaughter. Slaughter is required to ensure the elimination of any persistently infected (carrier) animals (Saiz *et al*., 2002; Alexandersen. *et al*., 2003).

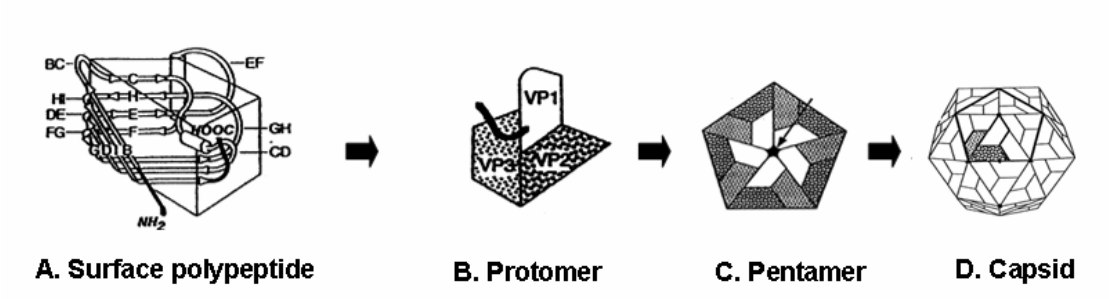

Figure 3 Schematic view of the surface structure of FMDV capsid proteins, the subunit assembly and the viral capsid.
A schematic tertiary structure of VP1, VP2, and VP3. (A) Surface polypeptide of FMDV capsid. (B) Arrangement of VP1-VP3 in a biological promoter; the location of the amino-terminal domain of VP1 is indicated by a black ribbon. (C) Arrangement of 5 protomers into a pentamer. (D) Structure of the capsid. A pentamer is outline in the capsid, and a protomer is indicated inside the pentamer.

Source: Saiz *et al*. 2002.

4. FMD vaccine

The FMD vaccines have been used in many parts of the world, particularly where the disease is endemic. Disease free countries have concentrated inactivated FMDV antigens kept in antigen bank which can be rapidly formulated into vaccine during an emergency (Statham *et al*., 2003).

The attempts to develop vaccines started in the early 1900s. The first practical inactivated vaccine was developed by Waldmann in 1937. Inactivation was with formaldehyde in the presence of aluminium hydroxide gel. The aluminium hydroxide functioned as an adjuvant as well as facilitating inactivation of the virus (Doel, 2003). Until early the 1950s, it was found that formalin-inactivated FMD vaccine caused the disease after application. In early 1960s, a new inactivant for FMDV was reported. It was N- acetylethyleneimine (AEI) which was an alkylating substrate. Other alkylating substrates were lately investigated. Currently, FMD vaccines are produced by growing live virus in BHK-21 cells. Virus is inactivated with binary ethyleneimine (BEI). BEI is produced by cyclization of bromo-ethylamine HBr (BEA) under alkaline condition (NaOH solution) (Bahnemann, 1975; Movat and Rweyemamu, 1997). Then inactivated virus is mixed with a buffer and adjuvant, either oil or aluminium hydroxide and saponin. It may be one (monovalent) or several of the serotypes (multivalent), but the strain(s) used should match the field strains (Davies, 2002).

Chemical inactivated FMDV vaccine has led to the successful eradication of the disease from the Western Europe (Brown, 1992). Although these killed vaccines have been effective, however, the recurrent problems are the development of the carrier state in some vaccinated animals, and relatively short lived immunity, a cold-chain to preserve vaccine stability (Salt, 1993). Another significant recurrent problems are that vaccine production factories and residual virus in the vaccine that have been implicated in disease outbreaks (McKenna *et al.,* 1996).

In vaccination policy, new vaccines are required to replace current vaccine. There are several basic rules that should be considered for the development of new FMD vaccine. The vaccines should include multiple B-cell and T-cell epitopes to produce humeral (antibodies) and cellular immune responses, particularly T helper response for antibody response. New vaccines should target systemic and mucosal immunity that may minimize chance of the establishing the carrier stage. In addition, vaccines should have capability to distinguish vaccination from infection (Domingo *et al*., 2002)

Since FMDV RNA extracted from FMDV or transcribed from full length cDNA copies of the genome is infectious. Therefore, vaccine manufacturing should not handing of virus (Domingo *et al*., 2002). To prevent contamination of FMDV, only capsid proteins are used. Recent advances in molecular biology promote a potential for alternative vaccines. The dissection of pathogen into their various components allows the development of specific subunit vaccines that are safer than whole pathogen vaccines (Streatfield *et al*., 2001). Empty capsids retain most of the immunogenic and antigenic property of viral particle (Saiz *et al*., 2002). The three dimensional arrangements of the capsid proteins provide the antigenic sites that elicit responses to vaccination or infection (Alexandersen. 2003). *In vivo* and *in vitro* studies by combining immunogenic, genetic and biochemical procedure reveal antigenic sites on capsid proteins summarized by Domingo *et al*. (2002) (Figure 3).

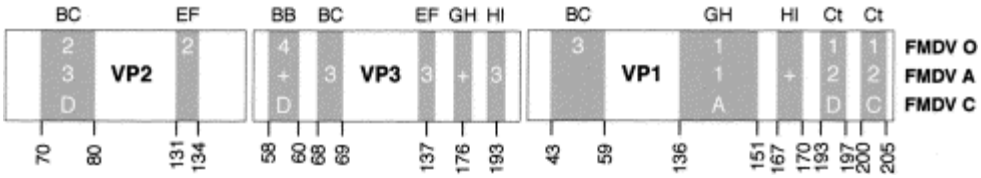

Figure 4 The location of the antigenic sites on FMDV surface capsid of serotypes O, A and C.

The three boxes (not drawn to scale) correspond to capsid proteins VP2, VP3 and VP1. The letters on top of each box indicate the antigenic loops (Ct is the carboxy-terminal region of VP1). Antigenic sites are indicated in gray boxes. The numbers and letters inside these boxes refer to site designation for FMD serotype O, A, and C; + indicates sites not specifically named in the original references. The numbers at the bottom indicate the amino acid positions (count individually for each protein) at which the different loops and domains are located.

Source: Domingo *et al*. 2002.

For serotype O, VP1 contains site one and site four epitope, while VP2 and VP3 contain one epitope each. Site one (also called site A in serotype A) comprises of a linear epitope at G-H loop (amino acid resdue 136-152), and epitopes at C-terminal of VP1 (amino acid residues 193-198 and 200-205) (Domingo *et al*., 2002). The VP1 fragments include a mobile loop between the βG and βH strands of VP1 (G-H loop) which protrudes from the viral surface (Domingo *et al*., 2002).

The G-H loop of VP1 has potentials to stimulate both B cell and T cell. The RGD motif (Arg-Gly-Asp at 145-147) of G-H loop is a critical part. It contains dual functions. First function is a B cell epitope and the second is binding with host receptor (integrin αVβ3) (Domingo *et al*., 2002). Early observations indicated that

either isolate VP1 or fragments derived from it carboxy-terminal half were the only viral capsid products capable of inducing neutralizing antibody (Saiz *et al*., 2002).

To express recombinant VP1 proteins, many systems are currently presented from various companies. Although most genes can be expressed in many systems, it is essential to determine which system offers the most advantage for the production of the recombinant protein.

5. Protein expression systems

The ideal system for expression is to produce most safe, biologically active material at the lowest cost (Daniell *et al*., 2001). Present expression systems can be categorized base on type of host cell into eukaryotic and prokaryotic expression system.

5.1 Eukaryotic expression systems

Eukaryotic expression systems are frequently employed for the production of recombinant proteins for therapeutics and research tools. Most commonly used are based on stably transfected adherent cells (CHO cell) or non-adherent cell (lymphoid cell). An efficient alternative is the infection of insect cells by recombinant baculoviruses. CHO, Sp2/0, and MEL cells are stably transfected cell lines. They give rise to production of fully glycosylated protein at variable product titers. In contrast, COS cells and insect cells (for baculovirus-mediation) are transient expressions. They rapidly produced incomplete glycosylated, and albeit biological active product (Geisse *et al*., 1996).

Mammalian cell cultures are preferred choice with respect to post-translational modification. The expressed proteins are identical to those of mammalian natural system.

The use of modified mammalian cell with recombinant DNA technology has the advantage that the resulting in productions are identical to those of mammalian. However, culturing is expensive and can only be carried out on a limited scale (Daniell *et al*., 2001). The cost is approximately 100 times that of microbial fermentation. This makes them impractical production systems for the large quantities of subunit vaccines (Streatfield and Howard, 2003).

Recently, plants have been used as recombinant biofactories to express a number of proteins including pharmaceuticals and potential vaccines (Koprowski, *et al.,* 1992; Mason *et al*., 1992; Haq *et al*., 1995; Gomez *et al*., 1998; Dozolme *et al*., 1999; Tacket and Mason. 1999). Plants have post-translational modifications similar to other higher eukaryotes and have ability to rapid scale-up and produce extremely large qualities. Using protein from plants reduces a fear of human pathogen contamination (Streatfield and Howard, 2003). However, plants have various ability to expressed protein of interest, time consume to produce stable expressed plant.

Another potential eukaryotic expression system is yeasts. They have been used as protein factories since the early 1980s for the large scale production of intracellular and extra cellular proteins of human, animal, and plant origin. Yeasts are suitable to express recombinant proteins. First, yeasts offer the ease of microbial growth and gene manipulation found in bacteria along with the eukaryotic environment. Second, yeasts have ability to perform many eukaryote-specific post-translational modifications, such as proteolytic processing, folding, disulfide bridge formation, and glycosylation. Third, relative to more complex eukaryotic expression systems, yeasts are economical. It usually gives higher yields, and is less demanding in terms of time and effort. Nevertheless, there are disadvantages in using yeasts for expression of some heterologous proteins. It mostly related to their inability to perform certain complex post-translational modifications, such as prolyl hydroxylation and amidation, phosphorylation and glycosylation (Cereghino *et al*., 1999).

5.2 Prokaryote expression systems

The expression of heterologous secreted proteins in bacteria has been studied both in gram positive and gram negative bacteria.

Gram-positive bacteria, for example, *Bacillus subtilis, Staphylococcus carnosus* have not achieved widespread acceptance. They excrete high levels of proteolytic enzymes that can cause extensive product degradation. In recent years, *Pseudomonas fluorescens* and *Ralstonia eutropha* have been used to produce remarkably high yields of recombinant proteins. A soluble organophosphohydrolase expressed in *R. eutropha* was high up to 10 g/L in high cell density fermentations. Although a high yield is expressed, there was little experience with this organism.

Currently, *E. coli*, gram negative bacteria, is widely used as a host organism to expression of secreted proteins. Conversely, the choice of bacterial strain, medium formulation, and the promoter and expression system are critical in determining protein yields. Remarkable yields of secreted proteins (in the 5–10 g/L range) have been obtained repeatedly. Such high yields are only obtained by high cell density fermentation using finely tuned expression systems. Nonetheless, it is reasonable to expect yields of at least 0.5–0.8 g/L for the vast majority of heterologous proteins. Many mammalian proteins can now be produced routinely in secreted form with yields in the gram/litre scale. Disadvantages that must be concerned are efficient secretion across the inner membrane, proteolytic degradation, incorrect disulfide-bond formation and aggregation into periplasmic inclusion bodies (Georgiou and Segatori, 2005).

Recombinant VP1 (rVP1) protein has been successfully expressed in many systems, such as in an insect cell (SF9) for baculovirus expression system and the 3-4 mg protein was utilized as a vaccine in swine. The data from this experiment showed 50% protection (Grubman *et al*., 1993). The rVP1 was also cloned and expressed yeast (*Pichia pastoris*). It could stimulate B cell and T cell immune response in

animals (Shi *et al*., 2006). The rVP1 expressed in *E. coli* (Kleid *et al.*, 1981; Grubman, *et al.,* 1993; Wang *et al.*, 2003) was applied to swine and cattle in ranges of concentration from 140 μg to 4 mg**.** It partially protects the animal from challenge with homologous FMDV, except an experiment that immunized with 140 μg of rVP1 could completely protect swine from challenge. Consequently, *E. coli* which was an economic expression system is interesting system to express rVP1.

Recently, *E. coli* expression system has been advance modified to solve its disadvantags. The pBAD Directional TOPO Expression system released by Invitrogen is designed to overcome the problems. The pBAD/D TOPO vector is a linear vector at a 4.4 kb in length. It contains pUC *ori* for maintenance in *E. coli*, and a kanamycin resistant gene to select positive transform colonies. The prominent advantages of this system are the high efficient transformation, a regulatory of protein expression, and simplified protein purification.

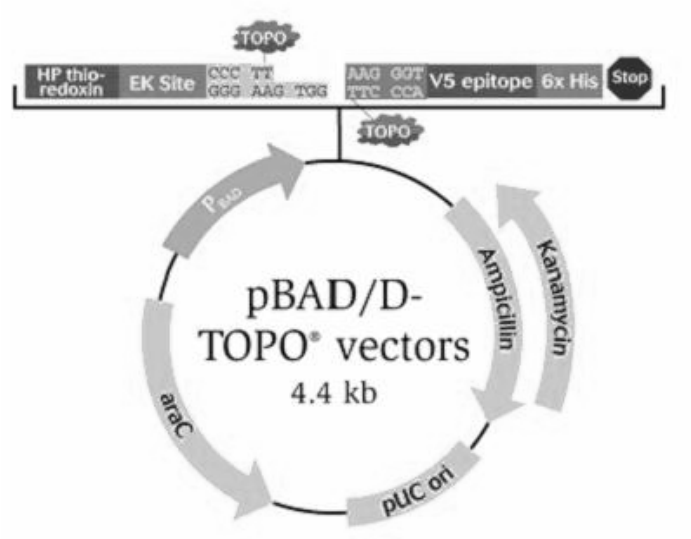

Figure 5 A pBAD/D TOPO vector

Source: Invitrogen. 2004.

The pBAD Directional TOPO@ Expression Kits (Invitrogen) utilize a highly efficient strategy to directional1y clone a blunt-end PCR product into a vector with no ligase, or restriction enzymes. The system use topoisomerase I from *Vaccinia* instead of ligase. The tyrosyl residue (Tyr-274) of topoisomerase I was covalently bond to a specific duplex DNA (5'-CCCTT-) at both ends of a linear vector, pBAD20/D-TOPO®. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the PCR product, and release topoisomerase (Invitrogen, 2004).

A directional joining of double-strand DNA, the vector has been modified by adding a 4 nucleotide (GTGG) overhang sequence to the TOPO@-charged DNA. The forward primer was added with 4 complementary bases (CACC) to the 5'-end as well. The overhang in the cloning vector (GTGG) invades the 5' end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Therefore, inserts are cloned in the correct orientation with efficiencies equal to or greater than 90%.

Expression of gene of interest by the pBAD/D-TOPO@ vectors is driven by the *araBAD* promoter. The promoter is indirectly induced by arabinose. In simply, arabinose induces araC gene to expresse. Subsequently, product from AraC gene stimulates araBAD promoter to transcripe the inserted gene. In the presence of arabinose, expression from pBAD/D-TOPO@ vectors is induced, while only very low levels of transcription are observed in the absence of arabinose. By varying the concentration of arabinose, protein expression levels can be optimized to ensure maximum expression. Uninduced levels are repressed by growth in the presence of glucose. In addition, the tight regulation of araBAD by AraC is useful for expression of potentially toxic or essential genes.

The vector also designed to increase solubility of a recombinant protein by adding thioredoxin at the N-terminal to a recombinant protein. Thioredoxin was originally isolated from *E col.* When used as a fusion partner, thioredoxin can

increase translation efficiency and, in some cases, solubility of eukaryotic proteins expressed in *E coli*.

The pBAD/D-TOPO@ vectors contains six histidine residues (6xHIS) at C-terminal to the inserted gene. This histidine patch has been shown to have high affinity for divalent cations. The recombinant protein can, therefore, be easily purified on metal chelating resins (Invitrogen, 2004).

MATERIALS AND METHODS

1. Virus and cell

A field isolate of FMDV serotype O, SEA topotype and baby hamster kidney - 21 (BHK-21) cell line were kindly provided by the Veterinary Diagnostic Laboratory, Faculty of Veterinary, Kasetsart University, Kampaeng Saen Campus. The BHK-21 cells were maintained in 25 ml flask containing Dulbecco's modified eagle medium (DMEM; Gibco) supplemented with 5% fetal bovine serum (HyClone® Coning) at 37 ^{o}C and 5 % CO_2. The FMDV stock was prepared by adding 100 µl of the virus stock to monolayer BHK-21 cells for 90 min. Afterward, virus was discarded and then 5 ml of DMEM supplemented with 5% fetal bovine serum was added. The infected cells were harvested when 60-75 % cytopathic effect (CPE) was detected. The virus was released from BHK-21 cell by frozen at -80^{o}C and thawed for 3 times and then centrifuged at 5,000 rpm for 5 minutes at 4^{o}C. The virus in supernatant was collected and stored at -80 ^{O}C until used (Mahy and Kangro, 1996).

2. Host strains and plasmid vectors

E. coli strains JM109 (Promega) and Top10 (Invitrogen) were used as competent cells. Plasmid pDrive (Qiagen) was used as a cloning vector. Plasmid pBad202/D-TOPO (Invitrogen) was an expression vector for VP1 gene of FMDV cloning.

3. Primers

Specific primers for the amplification of a whole VP1 gene of FMDV serotype O were designed using nucleotide sequences of FMDV serotype O from GeneBank. The forward and reverse VP1 primers contained restriction sites for enzymes *Bam*HI and *Xba*I, respectively. Additionally, the forward primer contained ribosome binding site (Kozak sequence) for enhancing translation process. The forward primer (FdVP1) sequence was 5’ GGG GGA TCC ACC ATG ACC ACC

TCT GCG GGT GAG TC 3', and reverse primer (RdVP1) sequence was 5' GGG TCT AGA CTA CTG TTT TGC GGG TGC CAC AAT C 3'. These primers gave the full-length of VP1 gene which was 633 bps in length. For cloning of whole VP1 gene into plasmid pBad202/D-TOPO®, the forward primer contained CACC sequence at 5' end (FbVP1 5' C ACC TCT GCG GGT GAG TC-3') which was very crucial for cloning into pBad202/D-TOPO® . The sequence of the reverse primer was RbVP1 5' ACT ACT GTT TTG CGG GTG CCA CAA TC-3'.

4. RNA extraction

Viral RNA was extracted from virus stock using acid-phenol-guanidinium-thiocyanate-chloroform extraction method (Sambrook *et al.,* 1989). Briefly, one hundred micro liters of supernatant was mixed with 500 µl of denatured solution and 50 µl of 2 M sodium acetate pH 4.5. Subsequently, one hundred fifty micro liters of each RNA phenol and chloroform were added and shaken for 5-10 minutes. The mixture was centrifuged at 13,000 rpm for 5 minutes and supernatant was then transferred to a new tube. This step was repeated for 1 to 2 times. The supernatant was added to 1.5 volume of isopropanol and placed in -80^{o}C freezer for 30 minutes before centrifuged at 13,000 rpm for 7 minutes. RNA pellet was air dried and resuspended with distilled water before reverse transcription was performed.

5. Reverse transcription (RT)

The RT was then performed to get a whole VP1 gene using FdVP1 and RdVP1 primers. The 25 µl of reaction mixture contained 1x Mg^{2+} free buffer, 1.5 mM Mg^{2+}, 0.25 µl RNasin (FINNZYMES®), 0.5 pmol of each primers, 1 unit of AMV reverse transcriptase (FINNZYMES®), 10µl of total RNA and 10 µl RNase free distilled water. The RT was conducted using a thermocycle (GeneAmp PCR system 2400, PERKIN ELMER.). The RT condition was started with 42 ^{O}C for 45 minutes for AMV-RT action and 94^{o}C for another 10 minutes.

6. Cloning of VP1 gene

6.1 Cloning of VP1 gene into plasmid pDrive

The cDNA from RT step was a template of VP1 gene in PCR step. The 50 µl of PCR mixture was comprised of 1x Mg 2^+ free buffer, 1.5 mM Mg 2^+, 0.5 pmol of each primer, 1 unit DyNAzyme (FINNZYMES®), 5 µl cDNA template and 36 µl distilled water. The PCR was performed using a thermocycle (GeneAmp PCR system 2400, PERKIN ELMER.). The PCR condition was started with pre-denaturation at 95°C for 5 minutes and followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds, and elongation at 72°C for 60 seconds and final extension at 72°C for 10 minutes (Sambrook and Russell, 2001). The PCR product was visualized by UV illuminator after electrophosis using 1 % agarose gel. The obtained PCR product was purified using QIAquick® Gel Extraction kit (Qiagen) according to the manufacturer recommendation.

The purified PCR products were subsequently ligated to plasmid pDrive. The ligation was performed at 4 °C for 16 hours using ligation mixture containing 1x ligation master mix, 4 µl of VP1 PCR products and 1 µl of plasmid pDrive. The ligation mixture was used to transform *E. coli* strain JM 109 by adding 10 µl of ligation mixture to 100 µl of competent cells and incubated on ice for 30 minutes. Subsequently, the transformation mixture was incubated at 42 °C for 1 minute and 4 °C for 10 minutes. One milliliter of LB broth was added to the transformation mixture and incubated at 37 °C for 60 minutes with 250 rpm constant rotation. The cells were harvested by centrifugation at 6,000 rpm for 1 minutes and the 1 ml of supernatant was discarded. The pellet was resuspended and spreaded on LB agar containing 100 µg/ml of ampicillin and 20 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and incubated at 37 °C for overnight. White colonies of *E. coli* were subcultured in LB agar containing 100 µg/ml of ampicillin and incubated at 37 °C for overnight. The recombinant plasmids were extracted to confirm the insertion by Polymerase chain reaction (PCR) technique. The selected *E. coli* clones were then

sequenced by dideoxynucleotide sequencing method at DNA Technology Laboratory, Nation Science and Technology Development Agency (NSTDA), Kamphaeng Saen, Nakhon Pathom. The nucleotide sequence was then aligned using DNASIS program (Toshiba).

6.2 Cloning of VP1 gene into plasmid pBAD202/D-TOPO®

The plasmid pDVP1 was used as DNA templates for cloning of VP1 into plasmid pBAD202/D-TOPO®. The PCR mixture contained 1x Mg^{2+} free buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.1 Tritonx 100), 1.5 mM Mg^{2+}, 0.5 pmol of each primer using in the RT step, 1 unit DyNAzyme EXT (FINNZYMES®), and 5 µl cDNA template. The PCR condition was pre-denaturation at 95°C for 5 minutes and followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds, elongation at 72°C for 60 seconds and final extension at 72°C for another 10 minutes. The PCR products were visualized by UV illuminator after electrophoresis using 1 % agarose gel (Sambrook and Russell, 2001).

The PCR products were purified using QIAquick® Gel Extraction Kit (Qiagen) and subsequently ligated to pBAD202/D-TOPO® plasmid (Invitrogen®) according to the manufacturer guide line to get pBADVP1. Briefly, purified PCR products were mixed with 1 µl of salt solution (1.2 M NaCl, 60 mM$MgCl_2$), 1 µl of plasmid and 1µl of distilled water. The ligation mixture was gently mixed and incubated at room temperature for 30 minutes. Three microliters of the ligation mixture were used to transform into One shot®TOP10 competent cells (Invitrogen®). Afterward, 250 µl S.O.C. medium was added into the transformed *E. coli* and shaken at 200 rpm for an hour before spreaded on LB agar containing 50 µg per ml of kanamycin. The present of inserted VP1 gene was determined by PCR technique (Sambrook and Russell, 2001).

7. Expression of VP1 protein in *E. coli*

7.1 Protein induction optimization

The inoculums were prepared by adding 100 µl of recombinant *E. coli* stock in 2 ml LB broth containing 50 mg/ml kanamycin and shaken at 200 rpm at 37°C for overnight. After that, each of five tubes containing 10 ml LB broth with 50 mg/ml kanamycin was added to 100 µl of inoculums. The tubes were shaken for another 2-3 hours until the optical density (OD_{600}) reach ~ 0.5. Then, the optimum condition for rVP1 expression was determined by adding different concentrations (0.2, 0.02, 0.002 and 0.0002%) of arabinose to recombinant *E. coli* culture medium. The cultures were then sampling every two hours. Subsequently, recombinant VP1 protein was partially purified using Ni-NTA bead as described by manufacturer (Qiagen) (Appendix B). The recombinant VP1 proteins were kept at -80°C for further verified by SDS-PAGE. The control samples were also conducted at the same condition but no adding arabinose.

7.2 Sodium dodecyl sulfate polyacrylamide gel electrophorsis (SDS-PAGE)

One microliter of induced recombinant *E coli* pellet was added to 5 µl of SDS sample buffer (250 mM Tris HCl, 20% glycerol, 4% SDS, and 0.006% bromophenol blue), boiled for 5 minutes and centrifuged at 5,000 rpm for 5 minutes. The supernatants were loaded into 12 % SDS-PAGE gel and electrophoresed for 50 minutes at 110 voltages. The gel was then stained with staining solution (0.01 % Coomasie Brilliant Blue, 50% methanol, 40% distilled water, and 10% acetic acid) for 10 minutes and subsequently destained with destaining solution (50% methanol, 40% distilled water, and 10% acetic acid).

7.3 Western blot analysis

The rVP1 proteins were first separated in 12 % SDS-PAGE gel and electro-transferred onto nitrocellulose membrane at 400 mAmp for 300 minutes. The

membrane was blocked with 5% skim milk at 4^{o}C for overnight. The membranes were then incubated with 1:1500 mouse anti-histidine antibody (Sigma) or 1:20 swine anti-FMDV hyperimmune serum for an hour at room temperature. Subsequently, the membranes were washed and incubated with 1:300 anti-mouse IgG peroxidase (KPL) or 1:2000 rabbit anti-swine IgG conjugated peroxidase (Sigma) for an hour at 37^{O}C. After washing, the membranes were incubated with 3 ml of 0.6 mg/ml diaminobenzidine (DAB, Sigma) containing 0.03% H_2O_2 for 5-10 minutes at room temperature. The rVP1 protein was visualized as a brown band on nitrocellulose membrane.

8. Animal Immunization

8.1 rVP1 protein production

The over night growth of recombinant *E. coli* were inoculated into 1 liter of LB medium at a concentration of 1: 100 and shaken at 200 rpm at 37^{o}C for 3 hours. Next, the cells were induced with arabinose at a 0.002 % final concentration, and shaken for another 6 hours. Finally, the induced recombinant *E. coli* were harvested by centrifugation at 6,000 rpm for 5 minutes. The cell pellet was stored at -20^{o}C and lysed before used.

8.2 Protein purification

The induced recombinant *E. coli* were lysed with lysis buffer pH 8.00 (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M urea), vigorously shaken and incubated for 2 hours at 4^{O}C. The lysed cells were sonicated for 10 minutes until the cell slurry changed from turbid to translucent. The cell debris was then separated by centrifugation at 5,000 rpm. The recombinant VP1 protein was purified using Ni-NTA bead as described by manufacturer (Qiagen). Briefly, Ni-NTA beads were first equilibrated with 3 volume of lysis buffer B at room temperature for 10 minutes. Buffer was then removed by centrifugation. An equal volume of cell lysate was added to Ni-NTA beads, shaken on ice at 200 rpm for 2 hours and centrifuged at

2,000 rpm for 2 minutes. The beads were washed with 3 volume of washing buffer C, mixed well by hand shaking, and centrifuged at 2,000 rpm for 2 minutes. The beads were then washed with 2 volume of washing buffer D. These washing steps were repeated for 5 times. The rVP1 protein was eluted from beads by 1 volume of elute buffer E. This step was repeated for 5 times. The purified rVP1 was verified by 12 % SDS-PAGE. Western blot analysis was performed to test the ability of rVP1 to interact with swine hyper-immune serum against FMD as described before (Sambrook and Russell, 2001). The purified protein was concentrated using acetone precipitating method. Briefly, two volumes of acetone was added to the protein, gently mixed and allowed the protein to sediment at -80 ^{O}C for over night. Afterward, the mixture was centrifuged at 13,000 rpm for 10 minutes. The pellet was allowed to air dry and stored at -20 ^{O}C before used.

8.3 Protein Measurement

VP1 protein was measured using modified Lowry protein assay kit (Pierce). Briefly, 40 µl of each sample was pipetted into a microplate well. Subsequently, 200 µl of Modified Lowry Reagent was added to each well and immediately mix the plate for 30 seconds. The microplate was incubated at room temperature for exactly 10 minutes. Afterward, twenty microliters of Folin-Ciocalteu reagent were added to each well, immediately mixed for 30 seconds and incubated at room temperature for 30 minutes. The amount of protein was determined at optical density 750 nm by ELISA reader (Rosys Anthos Lucy2). The BSA standard was simultaneously performed as a control.

8.4 Rabbit immunization

Eleven male New Zealand white rabbits (2-3 months, approximately 3 kgs) were separatly reared. They were randomly divided into three groups: group A was immunized with 300 µg of purified rVP1, group B was 500 µg immunization, and group C was immunized with 500 µl of protein from non-transformed *E. coli*. Group A and group C had three rabbits for each group and group B had five rabbits. Each

dosage of purified recombinant VP1 protein was vigorously mixed with the equal volume of complete Freund's adjuvant (CFA) for first dose or incomplete Freund's adjuvant for booster (Harlow and Lane, 1988). Each group was immunized at 1, 14, 28 and 42 days of the experiment. The rabbits were bled at day 0, 14, 28, 42, and 56 for post immunization (P.I.).

9. Anti VP1 antibody detection

9.1 Enzyme linked immunosorbent assay (ELISA)

Indirect ELISA procedure was performed as suggested by OIE (Office International des Epizooties) with some modification (OIE, 2004). The microplates (MaxiSorp, Nunc) were coated with inactivated FMDV diluted (1:2000) coating buffer to get the final intensity appropriate for detection by ELISA reader. The plate was incubated overnight at 4^{O}C. Sera were prior adsorbed with lysate of BHK cells in DMEM containing 5% fetal calf serum culture medium at a dilution 1:200. The sera were allowed to adsorb for 1 hour at 37^{O}C. Subsequently, 100 µl of each adsorbed sera was added into each well and incubated at 37 ^{O}C for 1 hour. The excess sera were washed out. Subsequently, 100 µl of 1:3000 goat anti rabbit IgG conjugated with horseradish peroxidase (Sigma®) was added into each well and incubated at 37 ^{O}C for 1 hour before washed out. Finally, 100 µl of Tetra-methylbenzidine (Sure Blue, KPL) substrate was added to each well and kept in dark for 30 minutes. The reaction was then stopped with 100 µl of 0.1 N H_2SO_4. The reaction intensity was measured by the ELISA leader (Losys Anthos lucy2) at the absorbence of 450.

9.2 Serum neutralization (SN)

The sera were inactivated at 56^{O}C for 30 minutes. Then, 50 µl of two fold dilution from each serum sample were added to the wells. The step was followed by adding 50 µl of 100 $TCID_{50}$ of FMDV into each well and incubated for 1 hour at 37 ^{O}C. The neutralizing ability of serum samples for FMDV infection were tested by adding 10^5 cells of BHK-21 cell into each well and incubated at 37 ^{O}C, 5% CO_2 for

another 24-48 hours. The cytopathic effect (CPE) of the BHK-21was recorded. In order to confirm the correct concentration of added virus, back titration was conducted by adding 100 µl of 100, 10, 1, 0.1 and 0.01 $TCID_{50}$ per ml to 10^5 cells of BHK-21 cell, respectively. Each dilution was repeated five times. The plate was incubated at the same condition as in the tested sera (OIE, 2004). Each serum sample dilution and back titration was 5 well repeated.

RESULTS

1. Cloning of VP1 gene of FMDV serotype O

1.1 Cloning of VP1 gene into plasmid pDrive

The PCR products of the VP1 gene of FMDV serotype O Thailand isolate were approximately 600 bps in length (Figure 6A). The PCR products were used to be ligated into plasmid pDrive before being transformed into *E.coli* strain JM 109. The recovered white *E. coli* colonies were subsequently determined for VP1 gene insertion by PCR technique. The PCR products from extracted recombinant plasmids showed the same size as those from a positive control. The restriction endonuclease assay was also performed to confirm the insertion. The digested products also had the size approximately 600 bps in length (Figure 6B).

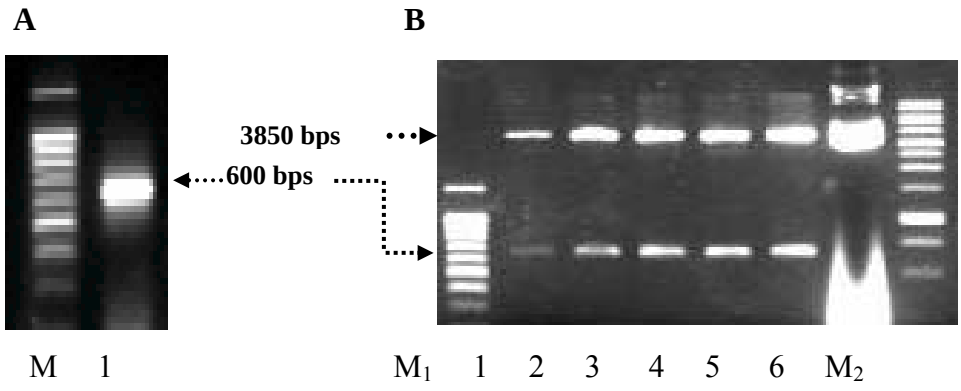

Figure 6 The PCR products and restriction fragment of VP1 gene.

The PCR products (A) and restriction fragment of VP1 gene (B) from RT-PCR technique and from restriction endonuclease assay, respectively. Figure A showed a single band of approximately 600 bps obtained from RT-PCR of virus RNA in 1% agarose (Lane 1). Figure 5B showed 600 bp band of VP1 gene and 3.8 kb band of pDrive vector (lane 1-5 in B) after recombinant pDVP1 was digested with restriction enzymes (*BamH* I and *Xba*I) compared with pDVP1 (lane 6). M and M_1 were 100 bp DNA marker while M_2 was 1 kb DNA marker.

The plasmid pDVP1 was sequenced, and revealed 633 base pairs in length. Its sequence showed 90 % homology with sequences of FMDV serotype O, SEA tpotype from Thailand, Lao, Malaysia, and Myanmar (Figure 7). The nucleotide sequence of VP1 gene was deduced to amino acid by ExPasy program and showed 89-91% identity to those SEA topotype isolated from Thailand, Lao, Malaysia, and Myanmar (Figure 8). The amino acid sequence of rVP1 protein was more identity to those from Thailand and Myanmar than those from Lao and Malaysia. The RGD motif was conserved at position 145-147. There were divergences of amino acid residue at N-terminal to the RGD motif: amino acid at position 139 from G to S, 141 from L to S, 142 from T to A and 143 from N to T. At C terminal, amino acid at position 158 was diverged from P to S.

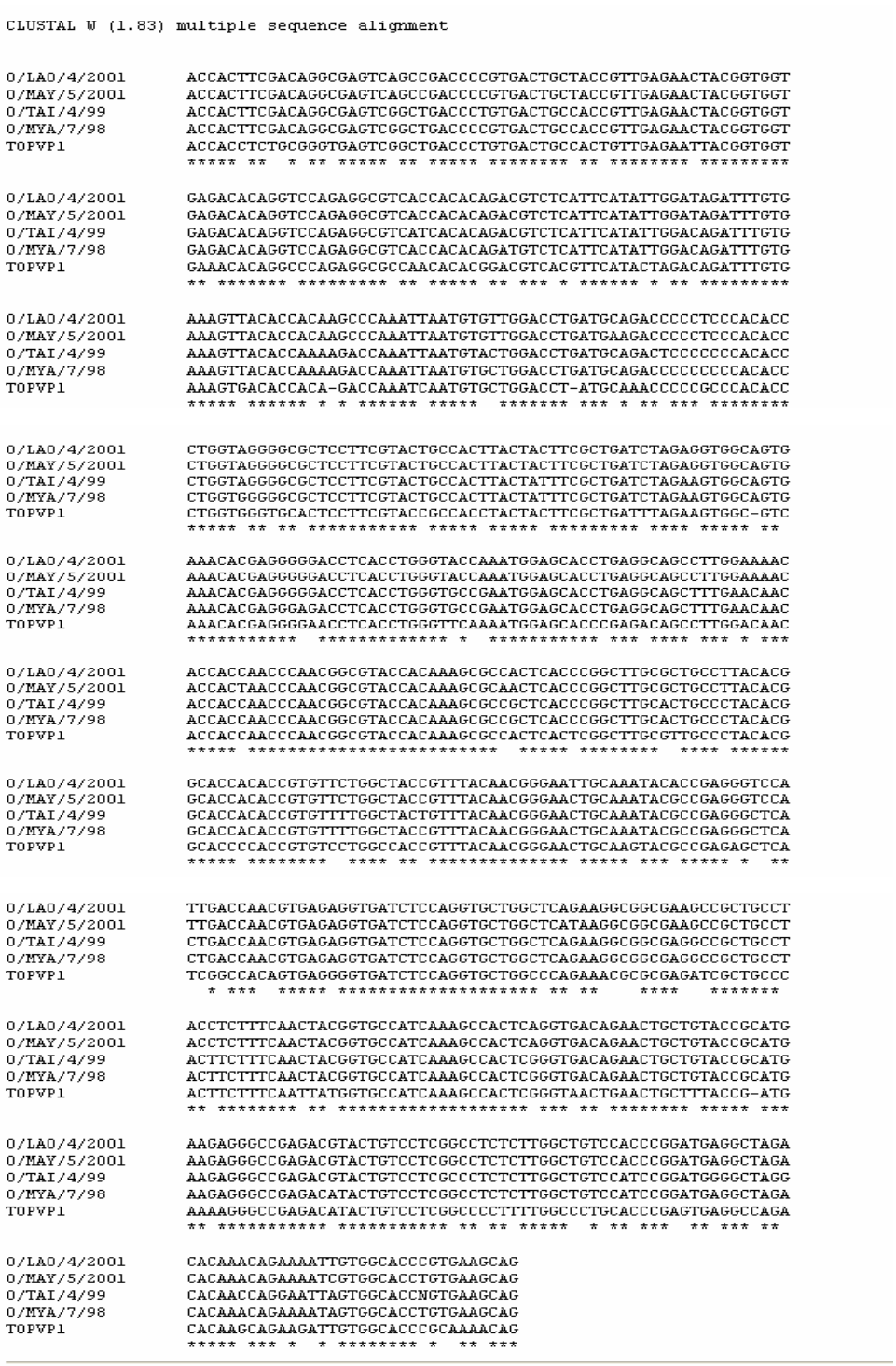

Figure 7 Nucleotide sequence alignment of recombinant VP1 gene.

Nucleotide sequence alignment of recombinant VP1 (rVP1) gene from TOPVP1 and those from Thailand (O/TAI/4/99), Myanmar (O/MYA/7/98), Lao (O/LAO/4/2001) and Malaysia (O/May5/2001).

```
CLUSTAL W (1.83) multiple sequence alignment


O/LAO/4/2001      TTSTGESADPVTATVENYGGETQVQRRHHTDVSFILDRFVKVTPQAQINVLDLMQTPSHT
O/MAY/5/2001      TTSTGESADPVTATVENYGGETQVQRRHHTDVSFILDRFVKVTPQAQINVLDLMKTPSHT
O/TAI/4/99        TTSTGESADPVTATVENYGGETQVQRRHHTDVSFILDRFVKVTPKDQINVLDLMQTPPHT
O/MYA/7/98        TTSTGESADPVTATVENYGGETQVQRRHHTDVSFILDRFVKVTPKDQINVLDLMQTPPHT
TOPVP1            TTSAGESADPVTATVENYGGETQAQRRQHTDVTFILDRFVKVTPQDQINVLDLMQTPAHT
                  ***:******************.***:****:***********: ********:**.**

O/LAO/4/2001      LVGALLRTATYYFADLEVAVKHEGDLTWVPNGAPEAALENTTNPTAYHKAPLTRLALPYT
O/MAY/5/2001      LVGALLRTATYYFADLEVAVKHEGDLTWVPNGAPEAALENTTNPTAYHKAQLTRLALPYT
O/TAI/4/99        LVGALLRTATYYFADLEVAVKHEGDLTWVPNGAPEAALNNTTNPTAYHKAPLTRLALPYT
O/MYA/7/98        LVGALLRTATYYFADLEVAVKHEGDLTWVPNGAPEAALNNTTNPTAYHKAPLTRLALPYT
TOPVP1            LVGALLRTATYYFADYEVAVKHEGNLTWVPNGAPETALDNTTNPTAYHKAPLTRLALPYT
                  *************** ********:**********:**:********** *********

O/LAO/4/2001      APHRVLATVYNGNCKYTEGPLTNVRGDLQVLAQKAAKPLPTSFNYGAIKATQVTELLYRM
O/MAY/5/2001      APHRVLATVYNGNCKYAEGPLTNVRGDLQVLAHKAAKPLPTSFNYGAIKATQVTELLYRM
O/TAI/4/99        APHRVLATVYNGNCKYAEGSLTNVRGDLQVLAQKAARPLPTSFNYGAIKATRVTELLYRM
O/MYA/7/98        APHRVLATVYNGNCKYAEGSLTNVRGDLQVLAQKAARPLPTSFNYGAIKATRVTELLYRM
TOPVP1            APHRVLATVYNGNCKYAESSSATVRGDLQVLAQKAARSLPTSFNYGAIKATRVTELLYRM
                  *****************:*.. :.*********:***:.*************:********

O/LAO/4/2001      KRAETYCPRPLLAVHPDEARHKQKIVAPVKQ
O/MAY/5/2001      KRAETYCPRPLLAVHPDEARHKQKIVAPVKQ
O/TAI/4/99        KRAETYCPRPLLAVHPDGARHNQELVAPVKQ
O/MYA/7/98        KRAETYCPRPLLAVHPDEARHKQKIVAPVKQ
TOPVP1            KRAETYCPRPLLAVHPSEARHKQKIVAPAKQ
                  *****************. ***:*::***.**
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Figure 8 Amino acid alignment of recombinant VP1 protein.

Amino acid alignment of recombinant VP1 protein with those from Thailand (O/TAI/4/99), Myanmar (O/MYA/7/98), Lao (O/LAO/4/2001) and Malaysia (O/May5/2001).

1.2 Cloning of VP1 gene into TOP10

The VP1 gene from pDVP1 was cloned in to pBAD/D-TOPO plasmid before being transformed into *E. coli* strain TOP10. The size of PCR products of the positive clones was approximately 600 bps of VP1 gene when visualized by electrophoresis technique (Figure 9).

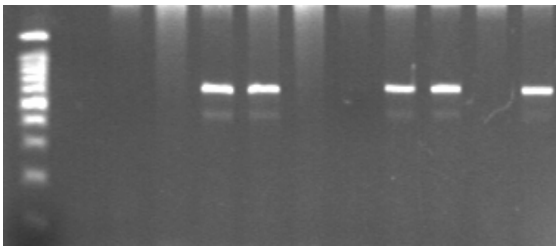

Figure 9 PCR products of the selected *E. coli* colonies.
PCR products of the selected *E. coli* colonies. Lanes 4, 5, 8, and 9 showed a specific band of approximately 600 bps in length. Lanes 10 and 11 were negative control and positive control, respectively. Lane M was 100 bp DNA marker.

2. Expression of recombinant VP1 (rVP1) protein

2.1 Optimization of protein induction

Five different arabinose concentrations (0.00002%, 0.0002%, 0.002%, and 0.2%) were tested for the optimization of protein induction. At 0.2%, 0.02% and 0.002% concentration of arabinose gave approximately the same amount of the rVP1 (Figure 10). For optimal time-course determination, samples were collected every 2 hours after adding 0.002% arabinose. The rVP1 protein was detected at 2, 4, 6, and 8 hours post induction (h.p.i.) and peaked at 6 h.p.i (Figure 11). Therefore, 0.002% of arabinose and 6 hours induction time were selected for the rVP1 production.

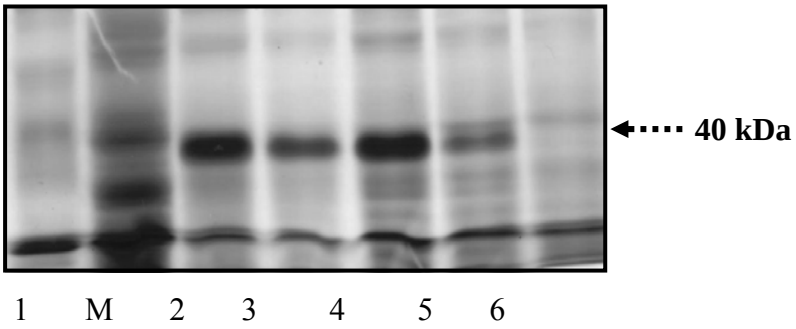

Figure 10 SDS-PAGE analysis of rVP1 from transformed *E. coli* induced with various arabinose concentrations.

12% SDS-PAGE analysis of rVP1 induction of transformed *E. coli* with various arabinose concentrations. Lane 1 was proteins from non-induced TOPVP1. Lane 2-7 were proteins from TOPVP1 induced with 0.2%, 0.02%, 0.002%, 0.0002%, and 0.00002% final concentration of arabinose, respectively. Lane M was protein marker (Invitrogen)

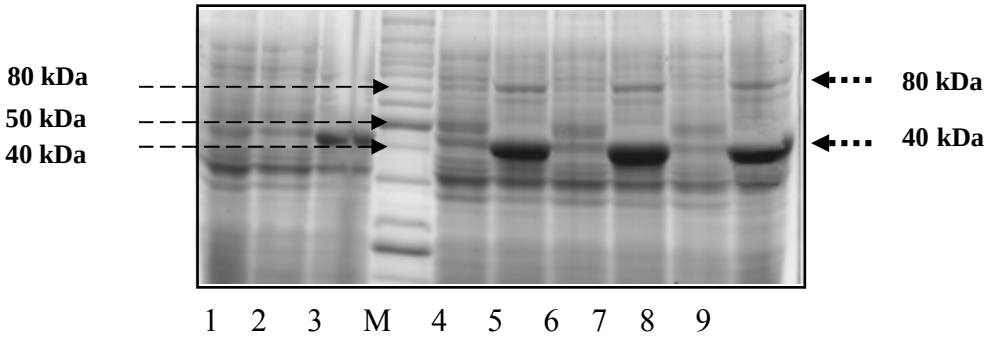

Figure 11 SDS-PAGE of time-course analysis for rVP1 protein expression.

12% SDS-PAGE of time-course analysis for rVP1 protein expression. Lane 1, 2, 4, 6, and 8 were proteins from non-transformed *E. coli* at 0, 2, 4, 6, and 8 hour post induction. Lane 3, 5, 7, and 9 were proteins from transformed *E. coli* at 2, 4, 6, and 8 hour post induction. Lane M was protein marker (Invitrogen).

2.2 Western blot analysis

The crude and purified rVP1 proteins were electro-transferred onto nitrocellulose membrane, subsequently the rVP1 proteins were incubated with swine anti-FMDV hyperimmune serum or mouse anti-histidine monoclonal antibody. For crude protein, there were 5 bands detected by both mouse anti-histidine monoclonal antibody and swine anti-FMDV hyperimmune serum which had molecular weight 72-100 kDa, 40 kDa, 33 kDa, 23 kDa and 17 kDa, respectively (Figure 12). There were also 5 protein bands when detected crude rVP1 protein with swine anti-FMDV hyperimmune serum. The bands were at the same molecular weight with those detected with anti-histidine monoclonal antibody. In contrast, the purified rVP1 protein gave only 2 bands which had the molecular weight 72-100 and 40 kDa. Protein of non-transformed *E. coli* showed negative result.

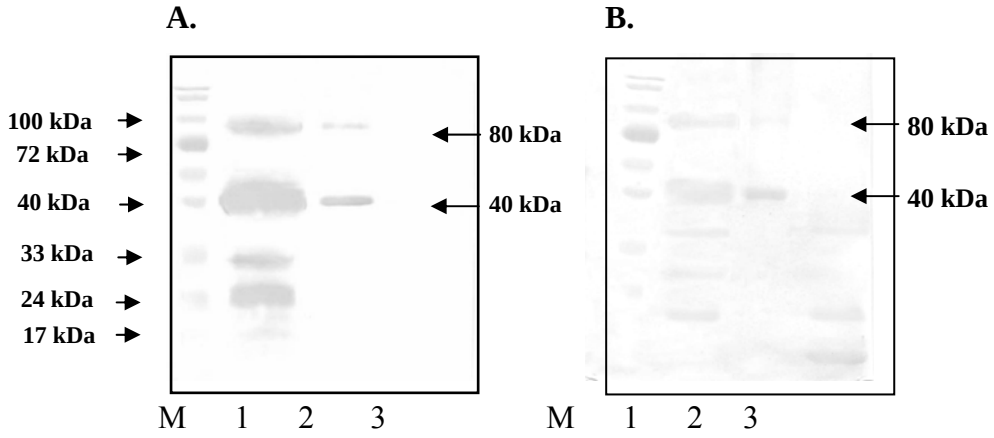

Figure 12 Western blot analysis of recombinant VP1 protein of FMDV.
Western blot analysis of rVP1 protein detected with mouse anti-histidine monoclonal antibody (A) and swine anti-FMDV hyperimmune serum (B). Lane 1 was crude proteins obtained from recombinant *E. coli*. Lane 2 was purified rVP1 from recombinant *E. coli*. Lane 3 was protein obtained from non-transformed *E. coli*. Lane M was protein marker.

3. Animal immunization

The precipitated rVP1 protein was mixed with the same volume of Complete Freund's adjuvant to get a homogenous mixture that had the concentration of 300 µg (group A) and 500 µg (group B) per 500 µl. The immunized rabbits were healthy after immunization until the fifth week when one rabbit in group C was diarrhea and death and one rabbit from group A was accidentally dead. The serum levels of antibody against rVP1 protein were determined using the ELISA technique. The mean OD values before immunization of all rabbits was 0.239 for Group A, 0.260 for Group B and 0.231 for Group C which were not significantly different among groups. For treatment groups, the OD values increased from 2 weeks post immunized until the end of experiment (Figure 13). The mean OD value of the two treatment groups was significantly higher than those from the control group every time point detected. However, the mean OD value between group A and B were not significantly different (Table 1). For serum neutralization (SN) test, rabbits immunize with protein from non-transformed *E. coli* showed cytopathic effect at each time point (Table2). For group A, SN titers were between 1:8 and 1:16 at the 2^{nd} week and gradually increased to the levels of 1:128 at the 6^{th} week and the titer remained the same until the end of the experiment. For group B, SN titers were 1:64 at 2^{nd} and were stable at this level until 8^{th} week post immunization.

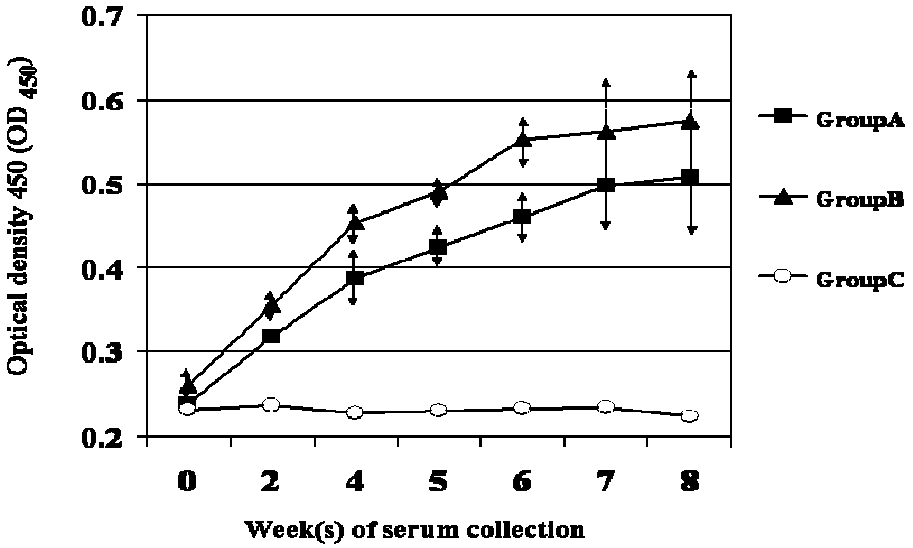

Figure 13 General Linear Model (GLM) of mean OD value of rabbit that immunized with rVP1 and control group.

General Linear Model (GLM) of mean value of reaction intensity of antibodies from sera of rabbit immunized with 300 µg of rVP1 (Group A), 500 µg (Group B), and control group (Group C) detected by indirect ELISA technique.

Table 1 ANOVA and Bonferroni Multiple Comparison of rabbit antibodies against FMDV. Mean value of reaction intensity were detected by ELISA technique.

Week(s) of serum collection	Mean ± SE (n=15)			*F*	df (Numerator, denominator)	*p*
	Group A	Group B	Group C			
0	$.239^{a}$ ±.0203	$.260^{a}$ ±.0429	$.231^{a}$ ±.0197	2.14	2, 24	0.139
2	$.319^{b}$ ±.0352	$.355^{b}$ ±.0429	$.237^{a}$ ±.0298	11.06	2, 24	0.00040
4	$.387^{b}$ ±.0837	$.454^{b}$ ±.0538	$.228^{a}$ ±.0113	23.18	2, 24	0.00001
5	$.424^{b}$ ±.0551	$.490^{b}$ ±.0467	$.230^{a}$ ±.0135	36.34	2, 24	0.00001
6	$.460^{b}$ ±.0772	$.553^{b}$ ±.0631	$.233^{a}$ ±.0143	40.41	2, 24	0.00001
7	$.498^{b}$ ±.1603	$.562^{b}$ ±.1439	$.235^{a}$ ±.0147	15.50	2, 24	0.00001
8	$.509^{b}$ ±.1677	$.575^{b}$ ±.1678	$.223^{a}$ ±.0173	14.29	2, 24	0.00001

Mean letter in the same row indicates significantly difference.

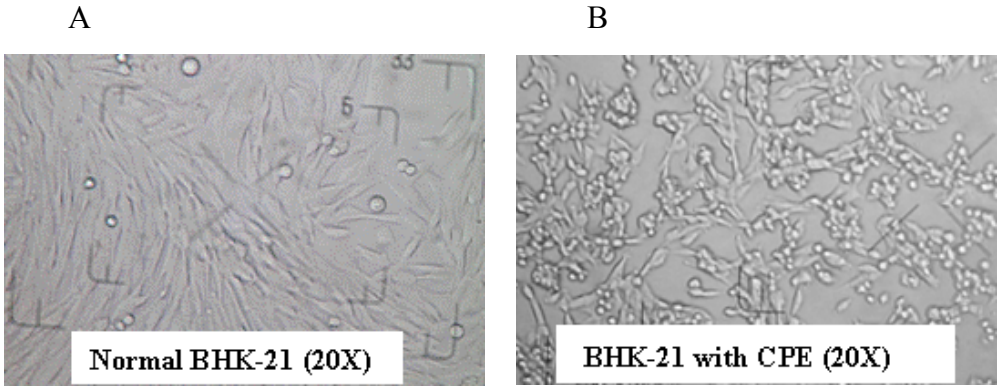

Figure 14 The morphology of normal and infected Baby Hamster Kidney 21 (BHK-21) cell line under light microscope.
The shape of normal BHK-21 cells was spindle, while, the infected BHK-21 cells showed cytophatic effect.

Table 2 Serum neutralization titer (SN titer) of rabbits immunized with 300 µg (Group A), 500 µg (Group B) of rVP1 and control (Group C). The FMDV-specific neutralizing antibody titer reported as a serum dilution yielding 50% CPE of BHK-21 cell.

Animals		Serum neutralization titer in week(s) post initial inoculation						
		0	2	4	5	6	7	8
Group A (300 µg)	No.1	0[c]	16[b]	32[b]	64	128[b]	128	128
	No.2	0[c]	8[b]	32[b]	*	-	-	-
	No.3	0[c]	16[b]	16[b]	64	64[b]	128	128
Group B (500 µg)	No.1	0[c]	64[b]	64[b]	64	64[b]	64	32
	No.2	0[c]	64[b]	64[b]	64	64[b]	64	32
	No.3	0[c]	32[b]	64[b]	64	64[b]	64	64
	No.4	0[c]	64[b]	64[b]	128	64[b]	128	64
	No.5	0[c]	64[b]	64[b]	128	64[b]	64	32
Group C (control)	No.1	0[c]	0[c b]	0[c b]	0[c]	0[c b]	0[c]	0[c]
	No.2	0[c]	0[c b]	0[c b]	*	-	-	-
	No.3	0[c]	0[c b]	0[c b]	0[c]	0[c b]	0[c]	0[c]

-[a] The numbers represent the lowest serum dilution that could protect BHK-21 from FMDV infection.

-[b] Animals were booster at week 2, 4, and 6 post initial inoculation with the same dose of antigen as in immunization

0[c] Rabbit sera cold not protect BHK-21 cells from FMDV infection

* Rabbit was accidentally died

DISCUSSIONS

1. Cloning of VP1 gene

The VP1 gene of FMDV serotype O, field strain was successfully cloned. The PCR product of VP1 gene was 633 bps when visualized in 1% agarose gel. The recombinant VP1 gene was aligned with other VP1 sequences and revealed 89% homology with sequences of FMDV serotype O, SEA topotype from Thailand, Lao, Malaysia, and Myanmar. Most of the different nucleotides were at the third nucleotide of triplet code (codon usage), which has less affects on a translation to amino acid (Lodish *et al*., 1999).

The deduced amino acid sequence recombinant VP1 protein was compared to those deduced amino acid sequences from GeneBank. The sequence showed 89-91 % homology of VP1 amino acid with those from FMDV serotype O, SEA topotype. The deduced amino acid sequence of recombinant VP1 protein was more identical to the sequences from Thailand (O/TAI/4/99) and Myanmar (O/MYAI/7/98) than those from Lao and Malaysia. This result indicated that FMDV circulating in western part of Thailand might be emigrated from Myanmar by smuggling movement of livestock across the border of Thailand (Ozawa, 1993). As a result, FMDV in western part of Thailand was closely related to that from Myanmar. The variation of the amino acid sequence of VP1 was seen around the G-H loop, however, the consensus amino acids of G-H loop, 144 (V), 147 (D), 148 (L), 149 (Q), and 154 (K), were conserved (Kweon *et al*., 2002). According to these mutations, the non-polar amino acids was changed to non-polar amino acids such as G to S at residue 139 and P to S at residue 158. The variation of these amino acids did not interfere the general structural features of the G-H loop (Logan *et al*., 1993; Lertwacharasarakul, 2006).

There are factors concerning in selecting the expression system. They were largely depends on the biochemical and biological properties of the protein of interest, the amount of recombinant protein required, as well as on the nature of the experiments (Geisse *et al*., 1996). VP1 protein of FMDV was successfully expressed

in many systems and the expressed proteins had capability to induce protective immunity. In current study, *E. coli* expression system was selected because it was cheap, fast and easy to manipulate. The plasmid pBAD 202/D-TOPO provides high ligation efficiency due to the attached topoisomerase at both ends of this linear plasmid (Invitrogen, 2004). The directional ligation of blunt end PCR product saves time for confirming gene orientation, so it needed only PCR technique to determine the inserted gene. Then, the selected colonies were ready for protein expression.

2. Expression of VP1 gene

According to the pBAD202/D-TOPO® expression system, the concentration of arabinose and the induction time can influence the amount of the expression of recombinant protein. In this experiment, the optimum concentration of arabinose and the optimum induction time were 0.0002% and 6 hours, respectively. The molecular weight of VP1 protein was calculated by summation of its amino acids (Adams, 2003) which was 26.93 kDa. The calculated molecular weight of rVP1 protein expressed using recombinant *E. coli* was 40 kDa due to the extra molecular weights of thioredoxin and 6 histidine included in the plasmid pBAD 202/D-TOPO. By SDS-PAGE, the rVP1 produceed from recombinant *E. coli* showed two prominent bands at 40 kDa and 80 kDa. The result was similar to rVP1 protein expressed in *E. coli* reported by Wang *et al*. (2003). By Western blot analysis, the rVP1 protein was positively reacted with both swine anti-FMDV hyperimmune serum and mouse anti-histidine monoclonal antibody. Crude rVP1 protein showed five bands when reacted with both swine anti-FMDV hyperimmune serum and mouse anti-histidine monoclonal antibody. However, purified rVP1 protein showed 2 bands of 40 kDa and 80 kDa when reacted with both swine anti-FMDV hyperimmune serum and mouse anti-histidine monoclonal antibody. This indicated that 33 kDa, 23 kDa and 17 kDa proteins might not be rVP1. The 40 kDa band of the rVP1 protein may be the monomeric form. The 80 kDa might be the dimer of the rVP1 which needs further study. These results were correlated with Wang *et al*. (2003) whose study shown that VP1 protein expressed in *E. coli* system contained both monomeric (29 kDa) and dimeric (58 kDa) rVP1. Naturally, VP1 protein contains cystein at residual 134 of

VP1 capsid protein of FMDV make a disulphide bridge to cystein 130 of VP2 (Logan, *et al*., 1993). There were two cystein amino acid residues (at 134 and 187) in this rVP1 sequence. Therefore, both cystein could form intra-molecular and intermolecular disulphide bond to create polymer rVP1.

3. Animal immunization

In the current study, rabbits were selected as a model to test ability of rVP1 to induce antibody response. The rabbits were divided into groups and each group contained at least three rabbits to reduce error from variation of individual (Halow and Lane, 1988). According to the pilot study in this experiment, rabbits could not induce immune response by immunization with 75 μg and 150 μg/animal of rVP1 (data were not shown). However, a wide dose range of antigen per does (50-1000 μg/animal) for rabbits was recommended by Halow and Lane (1988). Thus, administration of the higher doses of a recombinant VP1 protein was tested. Rabbits were immunized with two concentrations of 120 μg/kg/dose for Group A (300 μg/injection) and 200 μg/kg/dose for Group B (500 μg/injection) produced Ab to rVP1 protein. The same dose of 120 μg/kg/dose successfully induced antibody production in swine (Wang *et al.,* 2003). However, a higher dose (333-500 μg/kg/dose) of rVP1 were used in stimulation of immune response in mice (Shi *et al*., 2006).

In present experiment, Freunds' adjuvant was selected to promote a strong antibody response because subunit vaccine was less immunogenic than traditional vaccine (Tizard, 2000; Aucouturier *et al*., 2001; Hagan *et al*., 2001). It was a most commonly used adjuvant for research work. Complete Freunds' adjuvant (CFA) was co-administration with immunized dose. The Incomplete Freunds' adjuvant (IFA) was used in booster doses to replace CFA because *Microbacterium tuberculosis* membrane in CFA was high toxic to animal tissue (Harlow and Lane, 1988). Cox *et al*. (2003) immunized swine with conventional vaccine combination with Freunds' adjuvant and Montanide ISA 206. They found that a typical of primary responses (a rapid IgM response) was prominent 3-5 days following vaccination which peaked

within 7-14 days post immunization. This IgM response in the second pig trial persisted throughout the trial period up to 169 days post vaccination. Such a prolonged activity was a result of the antigen depot effect cause by the oil adjuvant used.

The ability of rVP1 protein for the induction of antibody was investigated by ELISA (OIE, 2004). The ELISA performed in this experiment was modified from indirect ELISA suggested by the OIE. The reaction intensity of serum from Group B were higher than those from Group A only 0.1 which was not significantly different when statistical analysis using ANOVA. However, they had been significantly different from those of the control group since the second week of the trial. The 120 μg/kg/dose (in Group A) and 200 μg/kg/dose (in Group B) gave the same antibody response may due to these two doses were in the same range of antigen to stimulate the same level of antibody. This indicated that the 120 μg/kg/dose was economic for rVP1 to be used as a vaccine. The potential may be increase or the dose may be reduced when the protein used with appropriated adjuvant(s). It is necessary to selected adjuvant(s) for food animal, animal species as well as economic cost. Singh and Hogan (2003) suggested that adjuvant used in veterinary should be low cost per animal. They may be mineral oil emulsion or aluminum hydroxide with additional compounds for immunopotentiation.

Since the VP1 protein contains neutralizing epitope at its G-H loop, SN technique was selected for *in vitro* testing for neutralizing activity of immunized sera. Unfortunately, animal challenging have to conduct at a highly restricted area and under the provision of the official authority. The rabbits from both treatment groups produced neutralizing antibody against FMDV which more than 1:32 of the SN titer that consider as a positive result (OIE, 2004). The SN titer from Group B could be positively detected one week earlier than those from Group A (week 4 and at week 5, respectively). This may be from a higher stimulated B-lymphocyte of Group B which was able to generate a high neutralizing antibody than in Group B. However, the SN titer from rabbits in Group A was higher than those in Group B at 7-8th week of experiment. This may be come from variation between individual rabbits or personal

error, which need more repeated SN test of these sera. The SN titer suggested that the higher dose had a chance to protect animal from infection faster than the lower dose. The SN titer from subunit vaccine was much lower than those from commercial vaccinated animal. Nevertheless, the recombinant protein(s) could protect animals from challenge (Moraes *et al*., 2003; Qian *et al*., 2004).

The positive SN titer in present trial supports the ability of rVP1 to be a subunit vaccine. However, the relationship between antibody titer detected either by virus neutralization tests or ELISA and protection against FMDV challenge depend on both the immunogen used for vaccination and the host (Berinstein *et al*., 2000). Consequently, further study of rVP1 in host animal including challenging is required to confirm the ability of rVP1.

CONCLUSION

The VP1 gene of FMDV serotype O Thailand isolate was cloned into plasmid pBad202/D-TOPO. The plasmid pBAD-VP1 was sequence and 89-91 % amino acid sequence homology with FMDV serotype O, SEA topotype and it was more identical to the sequences from Thailand and Myanmar than those from Lao and Malaysia. A recombinant VP1 protein was successfully expressed in *E. coli* expression system. The optimum concentration of arabinose and induction peroid for the expression of rVP1 protein was 0.002% and 6 hour. There might be two forms of rVP1, a monomer at (40 kDa) and an 80 kDa form. These two forms interacted specifically with swine anti-FMDV polyclonal antibodies and anti-histidine IgG.

The recombinant protein VP1 at doses 120 μg/kg (300μg/injection) and 200 μg/kg (500μg/injection) was capable to stimulate antibodies response which could detect by ELISA technique and serum neutralization test. It indicated that rVP1 expressed from TOPVP1 has a potential to be a subunit vaccine. Since there was no difference between the two treatments groups, the 120 μg/kg/dose of rVP1 was economic dose for vaccination. However, a high may be suitable when rapid protection is required. Nevertheless, the experiment was conducted in rabbit, so it need further study in host animal.

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APPENDIX

APPENDIX A

APPENDIX A

Chemical Reagents and Substances

1. Buffers for Protein Purification (Qiagen)

Lysis buffer B (1 liter):

100 mM NaH_2PO_4;13.8 g NaH_2PO_4 H_2O (MW 137.99 g/mol)

10 mM Tris Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 8.0 using NaOH

Washing buffer C (1 liter):

100 mM NaH_2PO_4;13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris-Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 6.3 using HCI

Elution buffer D (1 liter):

100 mM NaH_2PO_4;13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris.Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 5.9 using HCl.

Elution buffer E (1 liter):

100 mM NaH_2PO_4; 13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris-Cl 1.2 g Tris base (MW 121.1 g/mol

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 4.5 using HCl

Remark : The pH of buffer should be adjusted immediately prior to use. Do not autoclave.

2. SDS-PAGE reagents

- 2x SDS-PAGE sample buffer

  2.5 ml of 4xTris HCl / SDS, pH 6.8 (250 mM Tris HCl)

  2.0 ml of glycerol (20% glycerol)

  0.4 g of SDS or 4 ml of 1 0%SDS (4% SDS)

  0.2 ml of beta- mercaptoethanol (2% of 2-ME)

  0.006% bromophonol blue

- 4x Tris HCl / SDS pH 6.8, buffer for stacking gel (250 ml)

  0.5M Tris HCl ; 15 g Tris HCl

  0.4% SDS; 1 g SDS

  200 ml distilled water

  Adjust pH using concentrated HCl , then add distilled water to 250 ml total volume

- 4x Tris HCl / SDS pH 8.8. buffer for separating gel (500 ml)

  1.5M Tris HCl; 91 g Tris Base

  0.4% SDS; 2 g SDS

  400 ml distilled water

  Adjust pH using concentrated HCI then add distilled water to 500 ml total volume

- 30% Acrylamide / Bis-acrylamide (bis-acrylamide acrylamide =1:36) to prepare 513.5 ml of solution:

  150 g acrylamide

  4.1 g Bis-acrylamide

  Add distilled water to 513.5 ml sterilize by filter and store at 4°C

- 10% Ammonium persulfate

  100 mg ammonium persulfate

  1 ml distilled water

- TEMED (N,N,N',N'-tetramethylethylenediamine) store protected from light at 40°C

- Glycine buffer

192 mM glycine

25 mM Tris base

0.1% SDS

- Coomasie Brilliant Blue stain (2 litres)

2 g Coomasie brilliant blue powder

1 litre methanol

200 ml acetic acid

800 ml distilled water

Stir for minimum 2 hours and filter through Whatman filter disc

- Destainning solution (100 ml)

225 ml methanol

10 ml acetic acid

225 ml distilled water

3. Immunoblotting reagents

- PBS buffer, pH 7.4 (1 litre)

8.0 g NaCI

0.2 g $K_2H_2PO_4$

0.2 g KCl

1.15 g Na_2HPO_4

Distilled water adjust to 1000 ml

- PBS-Tween buffer

add to final concentration of 0.5% Tween 20 (Sigma®)

- Blocking agent

5% skim milk in PBS- 0.5 %Tween buffer

- Serum diluting agent

2% skim milk in PBS- 0.5 %Tween buffer

- DAB (Sigma®) substrate

  6 mg of DAB

  10 μl of H_2O_2

  990 μl of sterilize water

- Transfer buffer (1 litre)

  25mM Tris; 3 g Tris base

  190mM glycine; 14.4 g glycine

  20% methanol ; 200 ml conc. methanol (water adjust to 1,000 ml)

APPENDIX B

APPENDIX B

The standard methods

1. Preparation of *E. coli* competent cells for transformation

1 Culture *E. coli* strain DH5α cells on LB agar plate at 37 °C overnight.

2 Pick up a large colony and culture in 1 ml of LB broth at 37°C overnight with vigorous shaking (~ 250 rpm).

3 From 500 µl of overnight culture, subculture to 100 ml of SOB medium containing 25 µg/ml kanamycin, incubate at 37°C until OD_{600} is 0.4 - 0.8 (approximately 3 - 4 hrs).

4 Store the culture on ice for 10 minutes.

5 Centrifuge at 4 °C, for 10 minutes at 3,000 rpm, discard the supernatant.

6 Gently resuspend the pellet in 33 ml of ice-cold TB and store on ice for additional 10 minutes

7 Centrifuge at 4°C, for 10 minutes at 3,000 rpm, discard the supernatant.

8 Gently resuspend the pellet with 2 ml of ice-cold TB, then add 7% DMSO (150 ul)

9. Aliquot the cell to ependorf tube each 200 ul and store at -70°C until use for transformation.

2. QIA quick gel extraction kit protocol

1 Exise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2 Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl)

3 Icubate at 50°C for 10 mins. To help dissolve gel, mix by vortexing the tube every 2-3 minutes during incubation.

4 After the gel slice has dissolve completely, check that color of the mixture is yellow

5 Add 1 gel volume of isopropanol to the sample and mix.

6 Place a QIAquick spin column in a provided 2 ml collection tube.

7 Apply the sample to the QIAquick column, and centrifuge for 1 minutes.

8 Discard flow-through and place QIAquick column back in the same collection tube.

9 Add 0.75 ml of PE buffer to QIAquick colume and centrifuge for 1 minutes.

10 Discard flow-through and place QIAquick column an additional 1 minutes at 13,000 rpm.

11.Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

12 To elute DNA, add 50 ul of EB buffer (10 mM Tris-HCl, pH 8.5) to the center of QIAquick column membrane, let the column stand for 1 minute, and centrifuge for 1 minutes.

3. <u>Phenol-Chloroform extraction of RNA and ethanol precipitation</u> (Sambrook and Russell, 2001)

1 RNA was extracted from 100 µl of viral stock that mixed with 500 µl of denature solution and 50 µl of 2M NaAc, was shaken for 5-10 minutes

2 Add RNA phenol 150 µl and chloroform 150 µl was shaken for 5 minutes.

3 Centrifuge the sample at 13,000 rpm for 5 minutes to separate the phases.

4 Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted a second time with same procedure.

5 Repeat 2-4 again.

6 Remove about 90% of the upper, aqueous layer to a clean tube, add isopropanol 550 µl and 0.5 µl of glycogen (20ng/ml), invert gently up side down and keep in – 80°C for 40 minutes

7 Centrifuge at 13,000 rpm for 10 - 15 minutes. Carefully decant the supernatant.

8 To wash the RNA pellet with 75% ethanol. Centrifuge at 13.000 rpm for 5 minutes. Decant the supernatant, and dry the pellet by air.

4. <u>SDS-polyacrylamide get electrophoresis (SDS-PAGE)</u>

Prepare the gel by the recipe as follow for 2 gels

- Seperating gel (12%)

H_2O	2.8 ml
4x Tris HCI/SDS pH 8.8	2.0 ml
30% Acry/0.8%bis-Acrylamide	3.2 ml
10% APS	26.6 µl
TEMED	5.3 µl

- Stacking gel

H_2O	3.05 ml
4x TrisHCI/SDS pH 6.8	1.25 ml
30% Acryl/0.8%bis-Acrylamide	0.65 ml
10% APS	25 µl
TEMED	5 µl

4.1 If not already in electrophoresis sample buffer, add an equal volume of 2X sample buffer to all samples and boil for 5 minutes.

4.2 Apply 20-25 µl (1 - 10µg total protein) of cell lysate to each well of a 0.75-1.0 mm thick gel.

4.3 Run electrophoresis (100 volt, 70 minutes, constant ampere)