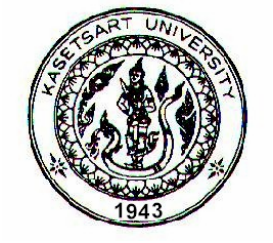


Thesis

EXPRESSION OF E2 PROTEIN OF CLASSICAL SWINE FEVER VIRUS AND ITS APPICATION AS SUBUNIT VACCINE

DAMRATSAMON SURANGKUL

GRADUATE SCHOOL, KASETSART UNIVERSITY

2006

THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Genetic Engineering)
DEGREE

Genetic Engineering
FIELD

Interdisciplinary Graduate Program
PROGRAM

TITLE: Expression of E2 Protein of Classical Swine Fever Virus and Its Appication as Subunit Vaccine

NAME: Miss Damratsamon Surangkul

THIS THESIS HAS BEEN ACCEPTED BY

Theerapol Sirinarumitr. **THESIS ADVISOR**
(Assistant Professor Theerapol Sirinarumitr, Ph.D.)

Gunjana Theeragool **COMMITTEE MEMBER**
(Assistant Professor Gunjana Theeragool, D.Agr.)

Sirirak Chantakru **COMMITTEE MEMBER**
(Assistant Professor Sirirak Chantakru, Ph.D.)

Amornrat Promboon **GRADUATE COMMITTEE CHAIRMAN**
(Assistant Professor Amornrat Promboon, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON 1 May 2006

Vinai Artkhayl **DEAN**
(Associate Professor Vinai Artkongharn, M.A.)

THESIS

EXPRESSION OF E2 PROTEIN OF CLASSICAL SWINE FEVER VIRUS AND ITS APPICATION AS SUBUNIT VACCINE

DAMRATSAMON SURANGKUL

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Genetic Engineering)
Graduate School, Kasetsart University
2006

ISBN 974-16-1579-5

Damratsamon Surangkul 2006: Expression of E2 Protein of Classical Swine Fever Virus and Its Application as Subunit Vaccine. Doctor of Philosophy (Genetic Engineering), Major Field: Genetic Engineering, Interdisciplinary Graduate Program. Thesis Advisor: Assistant Professor Theerapol Sirinarumitr, Ph.D. 111 pages.
ISBN 974-16-1579-5

Classical swine fever (CSF) is a contagious viral disease of swine caused by the CSF virus (CSFV). This disease is one of the most important agents causing economic losses to swine industry in Thailand. Modified-live virus (MLV) vaccine has been implemented to control CSF. This vaccine producing method is very expensive and inefficiency to produce high quantity of vaccine. Moreover, infected swine cannot be distinguished from vaccinated swine. It is important to develop a safer and more efficacious vaccine such as a nonreplicating E2 subunit vaccine. In the present study, E2 gene has been cloned and its protein is produced in *E.coli* by the pBAD directional TOPO™ expression system. The recombinant E2 gene is successfully cloned and expressed. Using western blot analysis, the recombinant E2 protein is clearly reacted with monoclonal antibody against E2 protein of CSFV. Finally, the recombinant E2 protein is used to immunize rabbits and serum from immunized rabbits are used to measure the antibody response. The result of this experiment showed that the recombinant E2 protein can induce anti-E2 specific antibodies in rabbits and may induce protective immunity in pig which may differentiate vaccinated pigs from infected pigs. The recombinant E2 protein may be a useful tool to eradicate CSFV from Thailand.

D. Surangkul
Student's signature

Thesis Advisor's signature

21 / April / 2006

ACKNOWLEDGMENTS

I would like to deeply express my sincerity to chairman of my thesis's advisor, Assist. Prof. Dr. Theerapol Sirinarumitr, for his kindliness, guidance, teaching, improvements and correctitude of my thesis. I also would like to express my grateful thanks to Assist. Prof. Dr. Gunjana Theeragool and Assist. Prof. Dr. Sirirak Chantakru, my thesis committee for their suggestion and comments.

This research was made possible by the most generous facilities provided in the Faculty of Veterinary Medicine, Kasetsart University and Veterinary Teaching Hospital. The staff of this establishment provided me the most generous help in every thing. I would like to thank Intervet (Thailand) Limited for providing anti-E2 IgG monoclonal antibody and CSF-SERO ELISA test kit.

Most of all, I am very thankful to my colleagues who share the same laboratory for their comment and assistance. Special thanks would also be directed to Miss Kannika Siripattarapravat, who shared her views and comments my research.

I would also like to acknowledge the financial support from the U.D.C. This thesis would never become reality without any intense encouragement, considerable support and understandable care of my family. All of them deserve thankfulness.

Damratsamon Surangkul

April 2006

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

µl	=	microliter
ml	=	mililiter
µg	=	microgram
mg	=	milligram
bp	=	base pair
kDa	=	kilodalton
pmol	=	picomole
mM	=	milimolar
M	=	molar
U	=	unit
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
dNTPs	=	Deoxynucreotide
PCR	=	Polymerase Chain Reaction
EtBr	=	Ethidium Bromide
UV	=	ultraviolet
SDS-PAGE	=	Sodium Dodecyle Sulfate Polyacrylamide Gel Electrophoresis
rpm	=	round per minute
ºC	=	Degree Celcius
E. coli	=	*Escherichai coli*

EXPRESSION OF E2 PROTEIN OF CLASSICAL SWINE FEVER VIRUS AND ITS APPLICATION AS SUBUNIT VACCINE

INTRODUCTION

Classical swine fever (CSF) is a highly contagious viral disease of swine. The disease is considered to have a particularly great impact on both the economical and epizootic situation. Classical swine fever virus (CSFV), the etiological agent of CSF, belongs to the Family *Flaviviridae*, Genus *Pestivirus*. The swine is the only natural host and is the significant source of the spreading of the CSFV. Direct contact between infected and susceptible pigs is the principal means of viral transmission. Infected swine may shed the virus before the onset of disease and continue to do so during the entire disease period. Thus pigs infected with virulent CSFV may shed large amounts of virus for duration of 10-20 days via oronasal and lacrimal secretions, urine, and feces, where as infections with low-virulence strains have short virus excretion periods. Consequently, virulent CSFV will usually spread faster in a herd and induce higher morbidity than low-virulent strains. Chronically infected pigs shed the virus continuously or intermittently till death. Pigs that recover from CSF generally shed virus until specific antibodies have developed (Van Oirschot and Terpstra, 1977).

Vaccination is employed in many countries to control outbreaks and has been used effectively in systematic and consistent programs to reduce disease outbreaks to a point where eradiation measures are feasible. Hog cholera lapinized virus (HCLV)

vaccine has played a key role in the control of epizootic CSF. In Thailand, modified-live virus (MLV) vaccine has been used to control CSF. The Department of Livestock Development, Minister of agriculture has been producing the vaccine against CSFV in rabbit using only Chinese strain. However, the amounts of the vaccine produced by the Department of Livestock Development hardly meet the requirement. In addition to protect animals from other strain of CSFV several strains of CSFV-vaccine MLV that have been imported to use in Thailand such as Chinese strain, GPE minus strain and Rom strain. The disadvantage of the use of MLV vaccine is that, infected pigs cannot be distinguished from vaccinated swine by RT-PCR or virus isolation (Van Rijn *et al.*, 1999). Thus, it is important to develop a safer and more efficient vaccine which will allow differentiating between vaccinated and naturally infected swine.

Subunit vaccine can be developed as an alternative to traditional vaccines. This vaccine has the advantage that it allows differentiation between vaccinated and infected pigs (Moormann *et al.*, 2000). The E2 protein the main envelop glycoprotein is capable of inducing immunity to protect CSFV infection (Hulst *et al.*, 1993). Vaccination with E2 protein express in insect cells protects swine against a lethal challenge with CSFV (Hulst *et al.*, 1993). An E2 subunit vaccine has been developed that is based on the expression of the E2 protein of CSFV in a baculovirus recombinant system (Van Rijn *et al.*, 1999). The transmembrane region of the E2 gene has been deleted, the protein is secreted to high levels into the supernatant of the insect cell culture. The baculovirus is inactivated and the E2 protein is then adjuvanted with a water-oil-water emulsion (Moormann *et al.*, 2000). The subunit

vaccine described in this study contains E2 protein produced in *Escherichia coli* (*E. coli*) cells by the pBAD directional TOPO™ expression system.

OBJECTIVES

1. To produce the recombinant E2 protein using pBAD directional TOPO expression system.

2. To determine the immunogenic potential of the recombinant E2 protein in stimulating the antibody production against CSFV.

LITERATURE REVIEW

Classical swine fever (CSF)

1. Clinical disease

Classical swine fever (CSF), also known as hog cholera, is a serious viral disease of swine characterized by fever, hemorrhages, ataxia, immunosuppression, abortion and results in high mortality. The disease may run an acute, subacute, chronic or unapparent course depending on a variety of viral and host factors of which the age of the animals, the virulence of the virus and the time of infection are of greatest importance (Dunne, 1975). Adult pigs usually display less severe signs of disease than young animals and have a better chance of survival. When virulent CSFV first appears in a herd, only a few pigs will show clinical signs of disease. Initially, the pigs may only appear drowsy or less active; if they are disturbed and made to stand, some will have arched backs and others may appear chilled. Still others may stand with drooping heads and straight tails. At this time, a reduced appetite is noticed. Later, it progresses to a marked anorexia exemplified by the pig nosing around in the feed for a short while before returning to its resting place. At the first sign of inactivity, fever can be recorded. Within 6 days of exposure to CSFV, the temperature of an affected animal may become higher than 42^{o}C, although values between 41^{o}C and 42^{o}C are more common during the course of the disease. Concurrent with the temperature rise is a corresponding drop in the leukocyte count. Total leukocyte counts of 9,000 to as low as 3,000 cells/mm^{3} of blood may be found

(Stewart, 1981). Early in the course of the disease, the eyes show a marked discharge associated with conjunctivitis, which may progress until the eyelids are completely adhered. Constipation commonly develops during the period of initial high temperature, followed by a severe, watery, yellowish gray diarrhea. Sick pigs become chilled and will huddle or pile on each other seeking warmth. It is not uncommon for pigs to vomit a yellowish fluid containing much bile. Convulsions may occur in a few swine, which usually die within hours or at most a few days after the convulsions begin. Hyperemia of the skin may occur concurrently with the initial temperature rise (Stewart, 1981).

As the disease progresses, more pigs become affected, and those that were sick first become gaunt and tucked up in the flank and have a characteristic weaving and staggering gait that appears to be directly related to a weakness in the hindquarters. This is usually followed by a posterior paresis. A purplish discoloration extending over the abdomen, snout, ears, and medial sides of the legs may occur near the terminal stage of the disease. Most pigs that suffer from acute CSF die between 10 and 20 days post-infection. In subacute CSF, pigs show less severe signs of disease and succumb within 30 days (Dunne, 1975). In an acute outbreak, a very large number of pigs can be expected to die after a severe illness. There are other forms of the disease. In the subacute and chronic forms of the disease, the symptoms are less severe but losses are due to mortality, poor performance and complications from other pathogens especially respiratory and enteric disease.

The virus readily crosses the placenta in pregnant sows (Dewulf *et al.*, 2001a; van Oirschot and Terpstra, 1977) and is present in most secretions and excretions, including the semen of boars (de Smit *et al.*, 1999; Hennecken *et al.*, 2000). The outcome of *in utero* infection depends upon the stage of gestation of the dam and the virulence of the virus involved. Piglets may be aborted or stillborn, may be persistently infected or may be normal and nonviraemic (Moennig and Plagemann, 1992).

2. Pathogenesis

Pigs are usually infected oronasally whereupon the virus multiplies in the epithelial crypts of the tonsil. Early targets for the virus are also low density granulocytic and monocytic cells. The virus may be carried to local lymph nodes and into the bloodstream for distribution throughout the body (Summerfield *et al.*, 1998; Susa *et al.*, 1992).

In acute infection, the distribution of virus through the host is characterized by a lymphatic, a viremic and a visceral phase. The tonsil is the primary target organ for virus multiplication. After initial replication, the virus is transferred, probably via the lymphatic vessels, to the lymp nodes draining the tonsillar region. The virus then reaches the efferent blood capillaries giving rise to an initial viremia and virus trapping in the spleen. The secondary target tissues produce large amounts of virus, resulting in high infectivity titers in the peripheral blood. Subsequently, CSFV replicates in other tissues of the immune system, such as visceral lymp nodes,

lymphoid structures lining the digestive tract and bone marrow. It is presumably late in the viremic phase that the virus invades parenchymatous organs. Generally, CSFV titers in lymphoid tissue are higher than in parenchymatous organs (Ressang, 1973)

CSFV has a predilection for reticular, endothelial and epithelial cells. The virus initially infects epithelial cells of the tonsillar crypts and later spreads into the surrounding lymphoreticular tissue. After a growth phase which occurs particularly in the lymp nodes, infection of organ epithelial cells is presumably mediated by cells of the reticulo-endothelial system (Ressang, 1973).

The time at which virus spread is completed mainly depends on the virulence of the virus. Highly virulent virus can be detected in most organs 5-6 days PI. In acute infection, severe thrombocytopenia and disturbances of fibrinogen synthesis develop. These disorders, in conjuction with degeneration of endothelial cells, cause the mortality from acute infection approaches 100%. Which mechanism is actually responsible for death is not well understood, but the severe circulation disturbances appear to be the most likely factor (Dunne, 1975).

The infection causes a severe leukopenia and immunosuppression, and there may be widespread thrombosis and/or endothelial damage. Gross pathological lesions are hemorrhagic diathesis and petechiation of organs (Moennig and Plagemann, 1992; Moennig *et al.*, 2003).

Since the virus is so innocuous *in vitro*, it has long been suspected that the serious lesions found *in vivo* must have an immunopathological origin. CSFV has been shown its ability to suppress host immune response through several mechanisms including avoidance of host immune attack and suppress immunological response. A soluble viral protein Erns in vitro testing of Erns protein showed that Erns protein at high concentrations was able to induce apoptosis in lymphocytes (Bruschke *et al.*, 1997).

3. Epidemiology

The disease is present worldwide and found worldwide. The global CSF situation has been reviewed (Edwards *et al.*, 2000). The known distribution of the disease in the last decade is shown in Figure 1 based on national reports to the Office International des Epizooties (OIE disease information: www.oie.int/eng/info/hebdo/a_dsum.htm). The disease was eradicated in the United States in 1978 after a 16-year effort. An eradication program is in force in the European Union (EU) countries. However, despite these measures, outbreaks of CSF continue to occur in the EU countries. In 1997, severe outbreaks of CSF occurred in a number of countries in Europe with large losses reported in Belgium, the Netherlands as well as Italy and Spain. In Western Europe, there has been a progressive eradication of CSF during the later decades of the 20th Century with vaccination banned in EU member states since 1990. However, the virus has been periodically reintroduced into domestic pigs via wild boar or imports from abroad (Gibbens *et al.*, 2000; Greiser-Wilke *et al.*, 2000). Parts of Central and Eastern Europe have followed

the EU model of non-vaccination, but CSF still remains present, particularly in countries with political instability. Wild boar numbers have dramatically increased in number and range in Europe since the Second World War. In some areas, e.g., parts of Germany, populations are now sufficiently large to be able to maintain CSF without reintroduction from domestic pigs or via swill feeding (Fritzemeier *et al.*, 1999, 2000; Moennig, 2000). To date there are several countries that are free from CSF include Australia, Canada, United Kingdom, Iceland, Ireland, New Zealand, the Scandinavian countries, Switzerland and the United States. Most CSF has been prevalent in Central and parts of South America for many years and vaccination is widely used. Outbreaks have also occurred in recent years in the Caribbean. Despite some setbacks, progress towards eradication is being made in Mexico. Countries in Asia can be regarded as endemic for classical swine fever although there is inadequate data to determine the incidence of the disease. The disease has been reported from Cambodia, Hong Kong, India, Korea, Laos, Malaysia, Myanmar, Nepal, Philippines, Sri Lanka, Thailand, and Vietnam. In 1996, outbreaks of the disease were also reported in Indonesia. The disease is important as it causes great losses to the pig industry in Asia (Kern *et al.,* 1999). CSF is endemic in much of Asia, where the greatest diversity of viruses is now found (Paton *et al.*, 2000). The situation of Africa is uncertain, but disease has been reported in Madagascar.

The virus can be spread via body fluid and secretions including urine and feces. As well as spreading directly from pig-to-pig, pig products including fresh, frozen and cured pig-meat can remain infectious to other pigs via the oral route (Edwards, 2000). The kinetics of spread between animals and herds has received

considerable attention in recent years (Klinkenberg *et al.*, 2002; Stegeman *et al.*, 1999a, b). During the Dutch epidemic of 1997/1998, spread by semen from an infected boar stud was also significant (Elber *et al.*, 1999). Airborne transmission of virus is probably occur only over a short distances and mainly within a holding. However, there has been concern that surrounding farms could be at risk from airborne spread during depopulation of affected premises (Dewulf *et al.*, 2000). Indirect transmission may occur via people, wild animals and inanimate objects, but the exact mechanisms whereby the virus spreads between neighbouring farms are poorly defined (Dewulf *et al.*, 2001). As with other List A diseases, many countries have insufficient resources to undertake adequate surveillance. This along with political/economic pressures against admitting the presence of the disease and the masking effect of vaccination are likely to result in an under-reporting of the true extent of the disease worldwide.

Figure 1 Worldwide distribution of classical swine fever since 1990, based on reported outbreaks

Source: Office International des Epizooties (2004)

There are approximately 1 million wild boar in EU Member States (Laddomada, 2000). At such levels, CSF virus eradication is extremely difficult and hunting to thin the population may be unsuccessful, or even counterproductive (unless carefully managed and targeted at young wild boar) by increasing the dispersal of animals and the consequent spread of disease. CSF in wild boar may be spread to domestic pigs by direct contacts in areas of extensive pig keeping (Biagetti *et al.*, 2001; Lowings *et al.*, 1999). The tendency for wild boar meat and trophies to be transported over long distances and to specialist restaurants provides a means of distant transmission to domestic pigs via the feeding of waste food.

Classical swine fever virus (CSFV)

1. Morphology of CSFV

The CSF virus is a member of the genus Pestivirus of the family Flaviviridae, and is closely related to the viruses of bovine viral diarrhea and border disease. There is only one serotype of classical swine fever virus. The virus is spherical, 40-60 nm in diameter, and consist of a tightly adherent lipid envelope covered with indistinct peplomers surrounding a spherical nucleocapsid with probable icosahedral symmetry.

CSFV is a small enveloped virus with a single-strand, 12.5 kb RNA genome of positive polarity. The CSFV genome contains a single open reading frame encoding an approximately 4,000 amino acid polyprotein which through cellular and

viral protease-mediated co- and posttranslational processing gives rise to the following 12 final cleavage products.

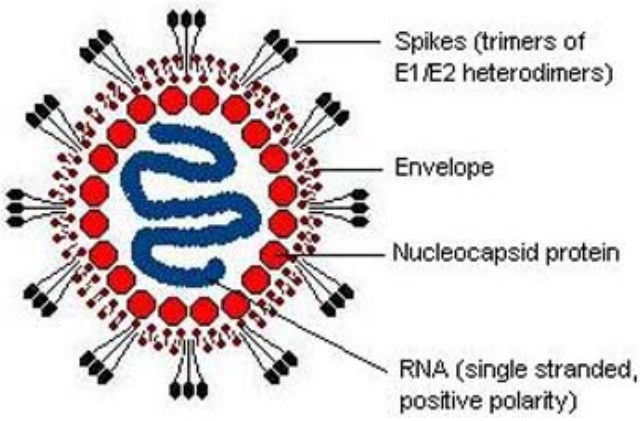


Figure 2 Structure of CSFV

Source: The University of Georgia College of Veterinary Medicine (2004)

The nucleocapsid is the protein structure surrounding the viral genome and is formed of repeating protein assembled around the nucleic acid. An icosahedron is a 20 sided solid with faces formed of identical equilateral triangles. The envelope is derived from the membranes of the host cell, although precisely which cellular membrane varies between viruses. Envelopment is not simply a passive process of picking up some membrane from the cell, since specific changes do occur to the membrane before it envelops the virus. Changes to membrane fluidity resulting from the preferential incorporation of specific lipids may be important. However, the most apparent change is the presence of viral proteins (seen as spikes) projecting through the envelope. Clearly, viral proteins must be present on the outside of the envelope

membrane in order to perform specifically viral functions such as binding to the host cell. These proteins are usually glycoproteins, with sugar groups attached to the polypeptide. The sugars make the protein locally hydrophilic, and are usually essential for function. All enveloped viruses have such proteins and, due to their nature and their position on the surface of the virion, they are usually highly immunogenic.

.

2. Genomic structures of CSFV

The classical swine fever genome is a single-stranded, positive-strand RNA of approximately 12.5 kb. At the 3'end of the viral genome, an untranslated region (3' UTR) consisting of about 228 nucleotides has been identified. The 3' UTR is most likely involved in initiation of the Pestivirus genome replication. Another cis-regulation site for the genomic replication is 5' untranslated region (5; UTR) at the 5' end of the genome, comprising approximately 373 nucleotides. The 5' UTR is also the site for initiation of translation of the CSFV genomes, in which an internal ribosomal entry site (IRES) is present (Le *et al.*, 1995). A single, long open reading frame (ORF) is flanked by the 3' UTR and the 5' UTR (Moormann *et al.*, 1996). Translation of the ORF leads to synthesis of a single polyprotein that is both co- and post-translationally cellular and viral protease, thus givings rise to 12 structural and non-structural (NS) viral polypeptides. The 12 proteins comprise 5 structure proteins (Npro, C, E1, E2, Erns) and 7 non-structure proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, NS5B)(Ruggli *et al.*, 1996).

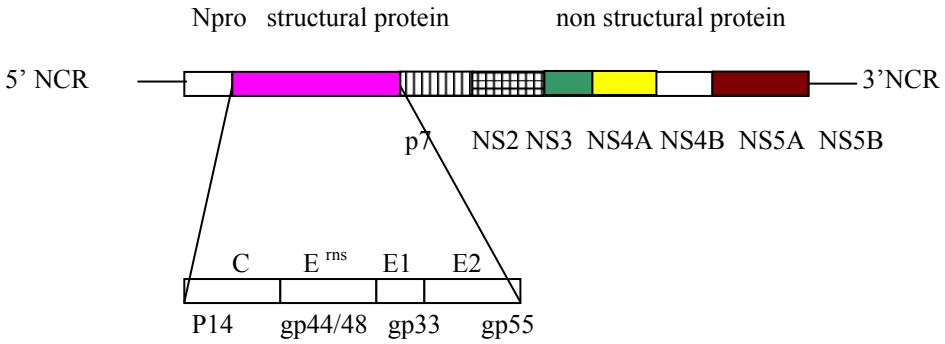


Figure 3 Genomic organization of CSFV

Npro is a papain-like cysteine protease responsible for the cleavage between Npro and capsid protein (C) (Meyer *et al.*, 2004). The gene for nucleocapsid protein precedes those of the E^{rns} protein (gp44/48); the latter is located at the surface of the virus as a homodimer but is also secreted from cells. The E^{rns} protein has unique ribonuclease activity (Schneider, 1993). The function of this enzymatic activity is as yet unclear. The E1 protein (gp33) is present in the viral envelope as an E1-E2 heterodimer, and the E2 (gp55), which is the most immunogenic protein of CSFV, is present as a homodimer and as a heterodimer with E1. The p7 protein is probably not incorporated in the virion, and the remaining C-terminal past of the open reading frame is coded solely for nonstructural proteins. The E2 protein has been found to be the main envelope glycoprotein capable of inducing immunity to infection. E2 protein immunization has been demonstrated the protecting effects against the challenge with swine fever virus (Muyldermans, 1993; van Rijn, 1994).

The NS3 was found to exhibit RNA-stimulated NTPase and/or RNA helicase activity (Warrener *et al.*, 1995). The NS5B protein is responsible for the replication of

CSFV genome (Steffen *et al.*, 1999). The pestivirus genomic replication consists of two consecutive processes. The replicase first binds to the 3' UTR and starts the genome replication, in which a minus RNA is produced with the plus RNA is produced with the novel minus RNA as a template (Meyer *et al.*, 1989). RNA-dependent RNA polymerase (RdRp) is the replicase of positive-strand RNA viruses. NS5B has been expressed and demonstrated to have the RdRp activity in hepatitis C virus (HCV)(lihmann *et al.*, 1997), Bovine viral diarrhea (BVDV)(Lai *et al.*, 1999) and CSFV (Steffen *et al.*, 1999). However, characterization of the RdRp activity of CSFV NS5B protein expressed in prokaryotic cells has not been reported yet. The NS3 protease is responsible for the cleavage of the viral polyprotein downstream of NS3 and requires NS4A as a cofactor (Tautz *et al.*, 1997). Furthermore, it possesses both helicase and NTPase activity (Warrener *et al.*, 1995) suggesting a role in RNA replication. The function of 4 nonstructural protein P7, NS2, NS4B and NS5A are not clear.

3. Biological properties of CSFV

Porcine kidney cells are used most frequently for CSF virus growth. Virus replication is restricted to the cytoplasm of the cell and does not cause a cytopathic effect. The first-progeny virus is released from the cells at 5-6 hours post-infection. Under single growth cycle conditions, there is an exponential increase in virus titer until 15 hours post-infection, after which virus production continues at a high level for several days. In cell cultures, CSFV spreads by means of the medium, by cytoplasmic bridges to neighboring cells, and from mother to daughter cells. Thermal and pH

resistance may vary among strain of CSFV. The higher the temperature, the quicker CSFV is inactivated. Inactivation of virus is partly dependent on both pH and temperature of the medium. In cell culture medium infectivity of virus is lost after 10 minutes at 60^{o}C, whereas in defibrinated blood the virus is not inactivated after 30 minutes at 68^{o}C. The virus is quite stable at pH 5-10. Above and below these pH values, virus is relatively rapidly destroyed. The inactivation rate below pH 5 is dependent on the temperature: at pH 4 a half-life of 260 hours was found at 4^{o}C and of 11 hours at 21^{o}C (Depner, 1992). Lipid solvents such as ether, chloroform, and deoxycholate quickly inactivate the virus. Fur disinfection, 2% sodium hydroxide is still considered most suitable. In pens the virus appears to be inactivated in liquid manure of pigs within few days. CSFV (initial titer of $10^{5.5}$ $TCID_{50}$ [median tissue culture infectious doses per mL) may survive for 2 weeks at 20^{o}C and more than 6 weeks at 4^{o}C. In pork and pork products, the virus can remain infective for months (Haas, 1995).

4. Diagnosis

Prevailing strains of CSF virus are of only moderate virulence (Koenen *et al.*, 1996; Williams and Matthews, 1988), making clinical diagnosis difficult especially in older animals. This increases the danger of delayed detection of primary cases as occurred in England in 2000 (Paton, 2002). The recent emergence of porcine dermatitis and nephropathy syndrome also complicates the diagnosis, since it can have a similar clinical appearance to CSF. Because the clinical signs of CSF are not pathognomonic, laboratory confirmation of disease is normally required, even for

secondary cases during large outbreaks (Koenen *et al.*, 1996). CSF often has an incubation period of some weeks, on a herd basis, requiring several cycles of amplification before it becomes clinically apparent. ‘‘Pre-clinical’’ detection would therefore be of enormous benefit to disease control. Fever is a very prominent sign in CSF and it would be extremely useful if pigs could be microchipped or screened en masse (for example using infra red devices) to select those with the highest temperatures for closer examination and sampling.

The conventional laboratory diagnostic methods are virus isolation and the demonstration of viral antigens in sections of frozen organs. These have been augmented by the use of antigen detection ELISA and RT-PCR (de Smit, 2000; McGoldrick *et al.*, 1999). The ELISA is a simple and rapid method for screening sick or pyrexic pigs and has the advantage that it can be used on large numbers of blood samples (Moser *et al.*, 1996). RT-PCR is more complicated and expensive but is also rapid and due to its greater sensitivity can be used on pooled samples and for preclinical diagnosis (Paton *et al.*, 2000). This method might be usable as a means of certifying that pigs at an abattoir were non-viraemic at slaughter as large numbers of blood samples are examined. An RT-PCR on meat-juices has also been investigated as an alternative to testing blood (Uttenthal, 2002). There is currently no practical means of wholesale screening of pig-meat imports, as the level of virus in meat is likely to be very low and the scale of required testing would be very large.

Infection with CSF virus is immunosuppressive and virus-specific antibodies are very slow to appear (Van oirschot, 1999). Thus, study routine sero surveillance is

not an effective method for early detection of newly introduced CSF (Crauwels *et al.*, 1999). Large-scale serological test is possible using commercially available ELISA kits. Unfortunately, the tests are not absolutely CSF-specific and can detect antibodies induced by other pestiviruses (bovine viral diarrhoea virus and Border disease virus), which occasionally infect pigs (Colijn *et al.*, 1997).

Control and Prevention

1. Control

More effort could be made towards a coordinated, global approach to the control of CSF and a world reference laboratory for the disease could be established. The new Council Directive 2001/89/EC provides the framework for the control policy within the EU (Anonymous *et al.*, 2001). Considering the progress in antigen detection methods the importance of serology in the control of acute outbreaks has somewhat decreased. However, serological diagnosis of CSF is still importance for surveys and the detection of hidden clusters of CSF. The virus neutralization test is the most sensitive and specific method for CSF antibody detection. Porcine serum samples are incubated with a CSF reference virus. In case the serum contains antibodies to CSFV the test virus will be neutralized. However,. cross neutralizing antibodies for ruminant pestivirus infections of pigs are often also registered by this test. Differential diagnostic for ruminant pestiviruses should therefore be carried out using a second neutralization test using ruminant pestiviruses. The neutralization test takes at least 2-3 days or longer if comparative testing is required and it is labour

intensive. Large numbers of serum samples are, therefore, processed using ELISA tests. Positive or unclear results should be retested using the neutralization test (Moennig, 2000). Swill feeding remains the significant source of classical swine fever into previously disease-free areas as the virus survives surprisingly well in a variety of meat products (Edwards, 2000). Swill feeding should therefore be banned or be strictly controlled to ensure that only properly heat-treated materials are fed. The public and industry also need to be informed of the risks from imported pig meat products. Corporate pig producers control multiple holdings and move large numbers of pigs on a regular basis. Eradication of CSF infected pigs is much more difficult in areas of high pig density such as occur in parts of North West Europe. Conventional live vaccines will lead to a marked reduction in clinical cases and in the levels of circulating virus (Anonymous, 1994). However, emergency vaccination using live attenuated CSF vaccine would result in an extended loss of trading status, since it prolongs the period during which a country remains under export restrictions due to its incompatibility with the use of serology to demonstrate disease freedom (Langedijk *et al.*, 2001)

2. Vaccination

Vaccination is one of the most important and cost–effective methods of preventing infectious diseases. Most attenuated vaccines are developed from the China-strain (C-strain) of lapinized CSF virus. The C-strain vaccines were and are still being used world-wide for the control of CSF in domestic pigs due to their efficacy to induce high titers of neutralizing antibodies and they are safe when used

on pregnant animals (Vlasova *et al.*, 2003). C-strain vaccinated pigs are also protected against infections with virulent CSF virus as early as five days after vaccination and are immune to the CSFV infection throughout their economic life. However, there is a severe disadvantage in using live attenuated vaccines against CSF. Vaccinated and field-virus-infected animals cannot be distinguished because the antibody pattern induced by the vaccine virus resembles that of reconvalescent animals (Vlasova *et al.*, 2003).

3. Development of subunit vaccines against classical swine fever infection

The recently developed E2 subunit CSF marker vaccines induce neutralizing antibodies against the E2 glycoprotein only (Hulst *et al.*, 1994; Van Rijn *et al.*, 1996). During an infection with field virus antibodies are produced against all viral proteins, although they do not all neutralize the virus. Consequently CSF antibodies which are not directed against the E2 glycoprotein should be indicative for an infection with CSF field virus. The accompanying discriminatory enzyme linked immunoassays (ELISAs) were designed to detect antibodies against the E rns g lycoprotein and, in case of a positive result, will confirm that the animal had been exposed to CSF field virus and could therefore be regarded as CSF infected (De Smit *et al.*, 2001).

When used as a prophylactic vaccine, marker vaccines induced good immunity against the clinical signs of CSF (Bouma *et al.*, 1999), although transplacental transmission of CSF virus could only be prevented after sows had received a booster vaccination (De Smit *et al.*, 2000). When test under conditions of

emergency vaccination, e.g. challenge before full immunity had developed, it was shown that CSF infection still occurred, although most challenge infections took a subclinical course with reduced virus shedding (Uttenthal *et al.*, 2001). In addition, transplacental transmission in pregnant sows could not be prevented after application of a single vaccine dose only (Depner *et al.*, 2001).

However, most highly developed to date are baculovirus expressed E2 subunit vaccines (van Rijn *et al.*, 1996, 1999), used in conjunction with marker serology based on detection of anti-Erns antibodies. Vaccinated animals can be distinguished from infected pigs using an ELISA based on a different viral protein as diagnostic antigens, such as the surface glycoprotein Erns or the nonstructural protein NS2.3 which is developed only in infected cases (Moormann *et al.*, 2000). .

Expression and purification system of recombinant proteins

1. In vitro expression of recombinant protein

Recombinant DNA technology provides a means of producing large amonts of viral proteins that can be purified readily and formulated into vaccines. Once the critical viral protein conferring protection has been identified, its gene (or, in the case of an RNA virus, a cDNA copy of the gene) may be cloned into one of a wide choice of expression plasmids and expressed in any of several cell systems. If the immunogenic viral protein of interest is glycosylated, eukaryotic expression systems

must be used so that the expressed protein is glycosylated and produced ideally in its proper conformation.

Useful eukaryotic expression systems include yeast cells, insect cells, and various mammalian cells. Yeast offers the advantage that there is extensive experience with scaleup for industrial production; the first vaccine produced by expression of a cloned gene, human hepatitis B vaccine, was produced in yeast. Insect cells offer the advantage of simple technology derived from the silk industry: moth cell cultures (or caterpillars) may be made to express very large amounts of viral proteins thrugh infection with baculoviruses carrying the gene of the virus of interest. The promoter for the gene encoding the baculovirus polyhedron protein is so strong that the product of a viral gene of interest inserted within the baculovirus polyhedron gene may comprise up to half of all the protein the infected moth cells or caterpillars make.

Mammalian cells offer the advantage over cells from lower eukaryotes in that they are more likely to possess the machinery for correct posttranslational processing, including glycosylation and secretion, of viral proteins.

Many different eukaryotic expression systems are available (Geisse *et al.*, 1996), ranging from the simple and cheap yeasts (Sudbery, 1996) to the mammalian cell lines (Geisse *et al.*, 1996) and transgenic animals (Echelard, 1996). Recently, plants have attracted attention as combined production hosts and oral vaccine delivery systems (Mason and Arntzen, 1995). Hepatitis B surface antigen (Mason *et al.*, 1992)

and Norwalk virus coat protein (Mason *et al.*, 1996) were expressed in tobacco and potato plants, and assembled into virus-like particles, which were shown to be immunogenic (Thanavala *et al.*, 1995). Oral immunisation, in this case feeding, with transgenic potatoes containing *E. coli* heat labile toxin subunit B (LTB) and the corresponding cholera toxin B subunit (CTB) elicited serum and local antibody responses in mice (Haq *et al.*, 1995; Arakawa *et al.*, 1998), and human clinical trials have been performed with the LTB-expressing potatoes (Tacket *et al.*, 1998).

Bacterial systems can express antigens at very high levels and are suitable for expressing vaccine antigens that do not require significant post-translational modification modifications. *E. coli* is the most commonly used bacterium for production of heterologous proteins, being easy to manipulate, genetically and physiologically well defined, and yielding high expression levels (Weickert, 1996). A multitude of vectors and strains are available, making it possible to design a suitable expression system. Expression of recombinant antigens in bacterial systems other than *E. coli* may sometimes be advantageous (Billman-jacobe, 1996). Salmonella typhimurium (Martine-Gallardo *et al.*, 1993; Liljeqvist *et al.*, 1996) and Bacillus brevis (Jakw *et al.*, 1995) are some examples of other bacteria that have been used for expression of antigens for vaccine production purposes. One particular feature in favour of Gram-positive bacteria is that the risk of contaminating lipopolysaccharides is avoided.

One potential drawback with prokaryotes as production hosts is that they are unable to carry out posttranslational modifications but other beneficial properties, and in particular the cost-efficient production systems, make bacteria the dominating hosts for production of subunit vaccine candidates**.**

2. <u>In vitro expression of recombinant protein in *E. coli*</u>

The pET TRX fusion system (Novagen, Madison, WI, U.S.A.) was chosen for the expression of E2 gene in *E. coli*. The BamHI-SalI fragment (covering the E2 gene) was derived from the cDNA clone of pE1 as described previously (Shiu, 1996) and was subcloned into the pET32c(+) vector. The resulting plasmid was designated as pET32cE2 and the expression of E2 gene was under the control of T7 lac promoter (Wong,1998). The E2 gene 1058 bp long PCR product was isolated by polyacrylamide gel electrophoresis (PAGE) (Dybczynski and Plucienniczak, 1998) and ligated into a pBluescript(-)(Stratagene) plasmid followed by transformation into DH5α *E. coli* strain. The cloned DNA fragment was then digested by NdeI and HindIII, and ligated into NdeI and HindIII digested pIGCmT7 expression vector. This plasmid is a derivative of the pIGDM1 plasmid (Mikiewicz, 1997), in which the target gene is placed under the transcriptional control of the T7 promoter. pIGCmT7 carries the gene encoding AGA and AGG tRNA. Ligated DNA was transformed into the expression host, *E. coli* BL21(DE3) strain.

The pBAD Directional TOPO Expression Kits utilize a highly efficient, cloning strategy to directionally clone a blunt-end PCR product into a vector for

regulated expression and simplified protein purification in *E. coli*. Blunt-end PCR products clone directionally at greater than 90% efficiency with no ligase, post-PCR procedure, or restriction enzymes required. In addition, pBAD/ D-TOPO vector contains the his-patch(HP) thioredoxin leader for increasing translation efficiency and solubility of recombinant fusion proteins. Expression of proteins in *E. coli* is driven by the *ara*BAD promoter (pBAD). The AraC gene product encoded on the pBAD/D-TOPO vectors positively regulates this promoter. Features of the vectors include *ara*BAD promoter (PBAD), N-terminal His-Patch thioredoxin, Directional TOPO Cloning site, C-terminal fusion tags, Kanamycin resistance gene, *ara*C gene encoding a regulatory protein and pUC origin of replication (Figure 4). The *ara*BAD promoter (PBAD) is used for the tight, dose-dependent regulation of heterologous gene expression. The N-terminal His-Patch thioredoxin increase the translation efficiency and solubility of heterologous proteins. The directional TOPO Cloning site is used for rapid and efficient directional cloning of blunt-end PCR products. The C-terminal fusion tag is used for detection and purification of recombinant fusion proteins. The kanamycin (pBAD202/D-TOPO) resistance gene is used for selection in *E. coli*. The *ara*C gene encodes a regulatory protein for tight regulation of the PBAD promoter. The pUC origin is used for maintenance in *E. coli*. In the presence of arabinose, expression of recombinant proteins encoded by pBAD vectors is induced whereas only very low levels of transcription are observed from pBAD vectors in the absence of arabinose (Lee, 1980; Lee et al, 1987)

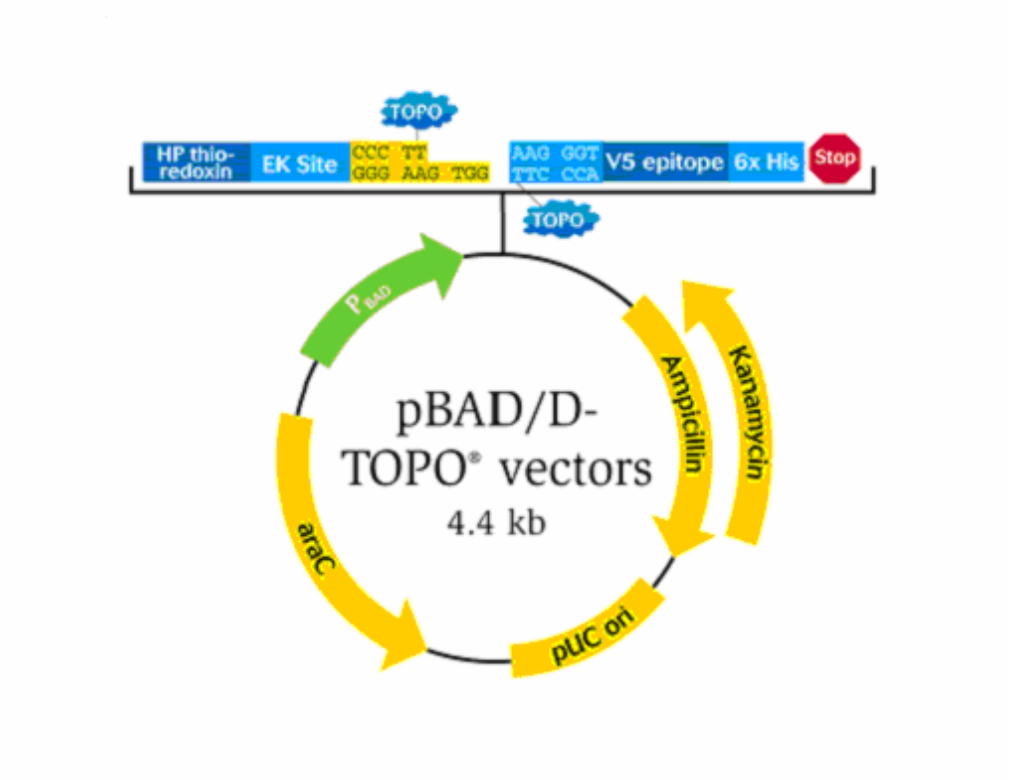


Figure 4 The pBAD Directional TOPO expression vectors

Source: Invitrogen Corporation (2006)

The 11.7 kDa thioredoxin protein is found in yeast, plants, and mammals, as well as in bacteria(Ref). It was originally isolated from *E. coli* as a hydrogen donor for ribonuclease reductase. The gene has been completely sequenced (Wallace and Kushner, 1984). The protein has been crystallized and its three dimensional structure determined (Katti *et al.*, 1990). When over expressed in *E. coli*, thioredoxin is able to accumulate to approximately 40% of the total cellular protein and still remains soluble. When used as a fusion partner, thioredoxin can increase translation efficiency and, in some cases, solubility of eukaryotic proteins expressed in *E. coli*.

The thioredoxin protein has been mutated to contain a metal binding domain, and is termed “His-Patch thioredoxin”. To create a metal binding domain in the thioredoxin protein, the glutamate residue at position 32 and the glutamine residue at position 64 were replaced with histidine residues(Lu *et al.*, 1996). To create the His-Patch thioredoxin folds, the histidines at positions 32 and 64 interact with a native histidine at position 8 to form a “patch”. This histidine patch has been shown to have high affinity for divalent cations (Lu *et al.*, 1996). His-Patch thioredoxin (HP-thioredoxin) proteins can therefore be purified on metal chelating resins (e.g. ProBond™). Expression of recombinant proteins with the N-terminal thioredoxin and/or the C-terminal peptide tags will increase the size of the recombinant protein(Table 1).

Table 1 Expected molecular weight increasing followed peptide tagging

Peptide Tag	Expected Size Increase (kDa)
N-terminal Thioredoxin	13 kDa
C-terminal V5, 6xHis	3 kDa

3. Purification of recombinant E2 protein

The Ni-NTA purification system contain a metal-chelating resin (Ni-NTA agarose) specifically designed to purify recombinant 6x His-tagged proteins. Ni-NTA

agarose is composed of Ni-NTA coupled to Sepharose CL-6B and offers high binding capacity and minimal nonspecific binding. This material has excellent handling properties for batch, column, and low-pressure FPLC. The high surface concentration of the NTA ligand is sufficient for the binding of approximately 5-10 mg of 6x His-tagged protein per milliliter of resin.

MATERIALS AND METHODS

1. Cells

E. coli strain Top10

(Genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G)

2. Cloning of the Gene Encoded E2 protein

2.1 Construction of E2 gene of CSFV and signal peptide

Signal peptide of TNF-α was prepared by Robust RT-PCR kit (Finzynes) from mRNA isolated from a porcine lymph node (Sambrook *et al.*, 1989). In brief, the first strand of cDNA are synthesized using oligo-dT as the primer and avian myeloblastosis virus reverse transcriptase in an extension for 50 minutes at 42 ^{o}C and a denaturation 5 minutes at 95 ^{o}C.

E2 cDNA are prepared from RNA isolated from commercial vaccine of classical swine fever virus using Robust RT-PCR kit (Finzymes). In brief, the first strand of cDNA are synthesized using hexamer as the primer and 1 unit of avian myeloblastosis virus reverse transcriptase was added in an extension for 50 minutes at 42 ^{o}C and a denaturation 5 minutes at 95 ^{o}C(Sambrook *et al.*, 1989) .

Primers for the E2 gene of CSFV and the TNF-α signal peptide were designed from the sequence data as report in GenBank. Primers for signal peptide were designed to construct DNA fragments with *Bam*HI restriction site at 5' end and *Eco*RI restriction site at 3' end. E2 gene primers were designed to contain *Eco*RI restriction site restriction site at 5' end and *Xba*I restriction site at 3' end.

E2 primer

Forward 5' – GGGGAATTCCGGCTAGCCTGCAAGGAAGA – 3'

*Eco*RI site

Reverse 5' – GGGTCTAGACTACCAGTACTGATACTCACCCTTAA – 3'

*Xba*I site

Signal peptide primer

Forward 5 ' - CGCGGGATCCACCATGGCCACCACGCTCTTC – 3'

*Bam*HI site

Reverse 5' – CGCGGAATTCGTCCTTGGGCCAGAGG –3 '

*Eco*RI site

Figure 5 Sequence of primers for amplified E2 and signal peptide gene

The reverse transcribed of E2 cDNA was then amplified by the polymerase chian reaction (PCR) using Proofreading DNA polymerase DyNAzyme EXT (Finzymes). The PCR reaction mixture (1X PCR buffer, 0.2 mM dNTPs mixture, 2.5 mM $MgCl_2$, 0.5 pmol of each forward and reverse primers, DyNAzyme EXT 1 U, and

template E2 cDNA) was amplified in the thermocycle (MJ Research, Inc.). The reaction mixture was preheated to 94 ^{o}C for 5 minutes, amplified by 35 cycles of 94 ^{o}C for 30 seconds, 55 ^{o}C for 50 seconds, 72 ^{o}C for 1.30 minutes, and finally incubated at 72 ^{o}C for 10 minutes. Amplified products analysed by agarose gel electrophoresis and viewed with gel documentation system (spectroline) (Sambrook and Russell, 2001).

The reverse transcribed of TNF-α signal peptide cDNAs was then amplified by the polymerase chain reaction (PCR) using Proofreading DNA polymerase DyNAzyme EXT (Finzymes). The PCR reaction mixture (1X PCR buffer, 0.2 mM dNTPs mixture, 1.5 mM $MgCl_2$, 0.5 pmol of each forward and reverse primer, DyNAzyme EXT 1 U, and template signal peptide cDNA) was amplified in the thermocycle (MJ Research, Inc). The reaction mixture was preheated to 94 ^{o}C for 5 minutes, amplified by 35 cycles of 94 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds, 72 ^{o}C for 30 seconds, and finally incubated at 72 ^{o}C for 10 minutes. Amplified products analysed by agarose gel electrophoresis and viewed with gel documentation system (spectroline) (Sambrook and Russell, 2001).

E2 gene fragments were ligated to plasmid pcDNA 3.1 (Promega) at *Eco*RI and *Xba*I restriction sites. The ligation reaction was used to transform DH5-α (Gibco) competent cell preparation (Appendix 1) (Sambrook and Russell, 2001). The positive clones were checked using PCR and restriction enzyme digestion. Thus, the positive colonies were scaled up and used for plasmid purification. Then signal peptide fragments were ligated to the plasmid E2 pcDNA 3.1 at *Bam*HI and *Eco*RI

restriction site to generate plasmid TNF-α-E2 pcDNA 3.1. The presence of TNF-α signal peptide and E2 gene were confirmed using PCR and restriction endonuclease assay. The positive colonies were scaled up and were sequenced. The sequencing results were analyzed using DNASIS program and Clustalw program to ensure that the sequence of E2 gene was correct and in frame at the fusion point.

2.2 Construction of pBAD Expression vector

Primers for amplification of E2 gene were designed using the sequence data of TNF-α-E2 pcDNA 3.1 plasmid with little modification. To enable directional cloning, the forward PCR primer contained the sequence CACC at the 5' end of the forward primer for ligation of PCR product with the GTGG overhang sequence of pBAD/D-TOPO vector. The reverse primer was designed to exclude the native stop codon of E2 gene for in frame ligation of PCR product to the c-terminal V5 epitope tag and polyhistidine region.

pBAD E2 Forward primer

5'- CAC CAT GCG GCT AGC CTG CAA GGA AGA - 3'

pBAD E2 Reverse primer

5'- GGG TCT AGA AAC CAG TAC TGA TAC TCA CCC TTA A- 3'

Figure 6 Sequence primer for amplified E2 gene for cloning into pBAD/D-TOPO vector

Proofreading DNA polymerase DyNAzyme EXT (Finzymes) was used in this experiment. The PCR reaction mixture (1X PCR buffer, 0.2 mM dNTPs mixture, 2.5 mM $MgCl_2$, 0.5 pmol of each forward and reverse primers, DyNAzyme EXT 1 U, and TNF-α-E2 pcDNA 3.1 plasmid) was amplified in the thermocycle (MJ Research, Inc). The reaction mixture was preheated to 94 ^{o}C for 5 minutes, amplified by 35 cycles of 94 ^{o}C for 30 seconds, 55 ^{o}C for 50 seconds, 72 ^{o}C for 1.30 minutes, and finally incubated at 72 ^{o}C for 10 minutes (Sambrook and Russell, 2001). The amplified products were analysed using agarose gel electrophoresis and were used to insert into pBAD/D-TOPO vector (Invitrogen, 2004).

2.3 Transformation to competent Top 10 cells

The recombinant plasmids were used to transform to TOP 10 competent *E. coli* (Invitrogen). Transformants were selected on medium plates containing kanamycin (50 μg/ml). The selected cloned were subcultured and grown overnight. The presence of the gene encoding for E2 protein has been confirmed by PCR using Taq DNA polymerase (Invitrogen, 2004)

3. Expression of the recombinant E2 protein

3.1 Concentration analysis of protein expression

The small expression culture was conducted to analyze the optimal concentration of arabinose. The arabinose concentration analysis of the level of

protein expression was performed and analyzed between a final concentration 0.00002 - 0.2 %. The single colony of the clone on agar plate was subcultured to 1 ml of LB broth containing 50 µg/ml Kanamycin and incubated overnight at 37 ^{o}C with shaking. Subsequently 0.1 ml of overnight cultured were inoculated into10 ml of pre warmed media with antibiotics, and grown at 37 ^{o}C with vigorous shaking (~ 250 rpm) until the OD600 between 0.5-0.7 was achieved. The sample (1 ml) was taken before induction, pelleted (centrifuged at 10,000 rpm for 5 minutes) and resuspended in 80 µl of 1x SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The sample was frozen at -20 ^{o}C until analyzed by SDS-PAGE. Expression of the recombinant protein was induced by adding arabinose to a final concentration 0.00002 – 0.2 %. The samples were collected at 4 hours, pelleted, and resuspended in 80 µl of 1x SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE (Invitrogen, 2004).

3.2 Time-Course analysis of protein expression

To analyze the optimal time of expression as well as the location of recombinant protein in the cells, a time-course analysis of the level of protein expression was performed and analyzed between 1-6 hours after induction. The single colony of the clone on agar plate was subcultured to 1 ml of LB broth containing 50 µg/ml kanamycin and incubated overnight at 37 ^{o}C with shaking. Thus 0.1 ml of overnight culture were inoculated 10 ml of pre-warmed media (with antibiotics), and grown at 37 ^{o}C with vigorous shaking (~ 250 rpm) until the OD600 between 0.5-0.7 was achieved. The sample (1ml) was taken before induction, pelleted (centrifuged at

10,000 rpm for 5 minutes) and resuspended in 80 µl of 1x SDS-PAGE sample buffer. Expression of the recombinant protein in *E. coli* was induced by adding arabinose to a final concentration of 0.002 % and then the bacterial were cultured for an additional 8 hours. The samples were collected every 2 hours, pelleted, and resuspended in 80 µl of 1x SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE (Invitrogen, 2004).

3.3 Growth of standard *E. coli* expression of the recombinant protein

The single colony of the selected clone of *E. coli* was subcultured in 10 ml of LB broth containing 50 µg/ml kanamycin and incubated overnight at 37 ^{o}C with shaking. The 10 ml overnight cultures were inoculated to 1 L of pre-warmed media containing 50 µg/ml kanamycin, and incubated at 37 ^{o}C with vigorous shaking at 250 rpm until the OD600 between 0.5-0.7 was achieved (approximately 2-3 hours). Expression of the recombinant protein was induced by adding arabinose to a final concentration of 0.002 % and grown for an additional 6 hours. Cells were harvested by centrifugation at 10,000 rpm for 5 minutes. The pellet was then washed with cold PBS and kept at -20 ^{o}C until use (Invitrogen, 2004) .

3.4 Growth of standard Top 10 *E. coli* without pBAD-E2 plasmid expression of control *E. coli* protein

The single colony of the Top 10 *E. coli* was subcultured to 10 ml of LB broth and incubated overnight at 37 ^{o}C with shaking. The overnight cultures (10 ml)

were inoculated to 1 L of pre-warmed media, and incubated at 37 ^{o}C with vigorous shaking (~ 250 rpm) until the OD600 between 0.5-0.7 was achieved (approximately 2-3 hours). Top 10 *E. coli* was induced by adding arabinose to a final concentration of 0.002 % and grown for an additional 6 hours. The cells were harvested by centrifugation at 10,000 rpm for 5 minutes. The pellet was then washed with cold PBS and kept at -20 ^{o}C until use (Invitrogen, 2004).

3.5 Determination of crude recombinant protein by Dot blot analysis

The crude extracted of *E. coli* containing plasmid pBAD-E2 plasmid was dot on nitrocellulose membrane and incubated with 5% skimmed milk for an hour and followed by incubation with 300 µl of 1:10 mouse anti-E2 monoclonal IgG antibody **(**Bommeli Diagnostics**)** in 2% skimmed milk for an hour at room temperature. The nitrocellulose membranes were washed with 0.15 M phosphate buffer saline (PBS; pH 7.4) for three times. Subsequently, the nitrocellulose membrane was incubated with 300 µl of 1:250 anti-mouse IgG conjugated with horseradish peroxidase (KPL) for 30 minutes at room temperature. The membranes were washed with 0.15 M PBS (pH 7.4) for three times and incubated with 300 µl of 0.6 mg/ml diaminobenzidine (DAB; Sigma**)** for 5-10 min at room temperature (Cochet *et al.*, 1998).

3.6 Determination of crude recombinant protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The pellet from 1 ml of recombinant Top 10 *E. coli* was resuspended in 80 µl of 1x SDS-PAGE sample buffer. The crude extracted protein was analyzed using 12 % SDS-PAGE, 120 volt, 60 minutes and strained with Coomassie brilliant blue. For Western blot analysis, proteins on SDS-PAGE were transferred on nitrocellulose membrane, incubated with 5% skim milk for 1 hour and incubated with 3 ml of 1:10 mouse anti-E2 monoclonal IgG antibody (Bommeli Diagnostics) for 1 hour. After incubation, membranes were washed with PBS (pH 7.4) for three times. Subsequently, the nitrocellulose membranes were incubated with 3 ml of 1:250 anti-mouse IgG conjugated with horseradish peroxidase (KPL) 30 minutes. The membranes were washed with 0.15 M PBS, pH 7.4 for three times and incubated with 3 ml of 0.6 mg/ml diaminobenzidine (DAB; Sigma**)** for 5-10 min (Cochet *et al.*, 1998).

4. Purification of the Recombinant Protein Using Ni-NTA

4.1 Preparation of cleared *E. coli* lysates under denaturing condition

The prepared cell pellets were thawed, resuspended in lysis buffer B (Appendix 1) at 5 ml per gram wet weight and sonicated on ice until the lysates

become translucence. The suspension was centrifuged at 10000 rpm for 15 min at 4 ^{o}C or room temperature to remove the cellular debris (Qiagen, 2001).

4.2 The purification of 6x His-tagged proteins from *E. coli* under denaturing condition

The 50 % Ni-NTA slurry 1 ml was added to 4 ml clear lysate and mixed gently by shaking (about 200 rpm) for 1 hour at room temperature. Subsequently, the lysate-resin mixture was centrifuged at 1500 rpm 2 min to remove the supernatant. The protein bound resin was washed 4 timed with 4 ml of washing buffer C (Appendix1). The polyhistidine tag fusion protein was eluted twice with 0.5 ml elution buffer D and followed by 4 times with 0.5 ml elution buffer E (Appendix 1). Each fraction were collected and analyzed by SDS-PAGE (Qiagen, 2001).

4.3 Determination of the recombinant protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The purified recombinant protein and *E. coli* protein were analyzed in 12 % SDS-PAGE, 120 volt, 60 minutes and strained with Coomassie brilliant blue. For Western blot analysis, proteins on SDS-PAGE were transferred on nitrocellulose membranes which were incubating with 5% skim milk and incubated with 3 ml of 1:10 mouse anti-E2 monoclonal IgG antibody (Bommeli Diagnostics) for 1 hour. After incubation, membranes were washed with PBS (pH 7.4) three times. Subsequently, the nitrocellulose membranes were incubated with 3 ml of 1:250 anti-

mouse IgG conjugated with horseradish peroxidase (KPL) 30 minutes. The membranes were washed with 0.15 M PBS, pH 7.4 three times and incubated with 3 ml of 0.6 mg/ml diaminobenzidine (DAB; Sigma) for 5-10 min (Cochet *et al.*, 1998).

5. Immunization the E2 protein to rabbits and detection antibody against the E2 protein by CHEKIT-CSF-SERO enzyme immunoassay (EIA) kit

5.1 Measurement protein concentration by modified Lowry protein assay kit

The purified recombinant protein and *E. coli* protein were measured protein concentration by modified Lowry protein assay kit (Pierce). In brief, 40 µl of each standard and recombinant protein samples were pipetted into a microplate well. Two hundred microliters of modified Lowry reagent were pipetted into each well at nearly the same moment. Immediately microplate was mixed for 30 seconds and incubated at room temperature (RT) for 10 minutes. Twenty microliters of 1X Folin-Ciocalteu reagent were pipetted into each well. Immediately, microplate was mixed for 30 seconds and incubated at RT for 30 minutes. The absorbance was measured at 650 nm using microplate reader (Tecan) (Pierce, 2005).

5.2 Immunized recombinant E2 protein to rabbit

The 9 rabbits were divided into 2 groups: the control and the treatment. In the control group, three rabbits were injected with purified protein of Top 10 without pBADE2. In the treatment group, six rabbits were injected with recombinant E2 protein (400 μg per dose). At days 0, 14 and 21 day, both groups of rabbit were injected intramuscular with purified Top 10 protein or purified recombinant E2 protein. Blood samples were collected from each rabbit at weeks 0, 2, 3, 4, 5 and 6 after injection.

5.3 Detection antibody against E2 protein by EIA kit

Rabbit serum from 9 rabbits was used to detect antibody against E2 protein by a competitive ELISA (CSF-SERO Kit; Bommeli Diagnostics), according to manufacturer recommendation. Briefly, Seventy five microliters of sample diluent was pipetted into each well of the microplate. Fifty microliters of the undiluted samples and controls was pipetted into the appropriate wells of the microtiter plate. The mixture was mixed briefly and incubated for 90 minutes at room temperature using a humid chamber. After the incubation, microtiter plate was washed with 300 μl of washing & dilution solution for 2 times at room temperature. The anti-CSFV-SERO-Conjugate (1:200) was added and incubated for 30 minutes at room temperature in a humid chamber. After the incubation, the microtiter plate was washed with 300 μl of washing & dilution solution three times. Substrate was added and incubated for 20-30 minutes at room temperature. The reaction was stopped by

adding 50 µl per well of Stopping solution at room temperature. The absorbance was measured at the wavelength of 405 nm. The difference in the OD between the negative and positive controls is ≥ 0.3. Each sample was repeated three times.

The inhibition by the positive control (INpos) is calculated by subtracting the OD of the reaction obtained in wells containing the positive control (ODpos) from the OD of the reaction obtained with the negative control (ONneg) (Bommeli Diagnostic).

$$INpos = ODneg - ODpos$$

The inhibition by the sample (INsample) is calculated by subtracting the OD of the reaction obtained with the sample (ODsample) from the OD of the reaction obtained with the negative control (ODneg) (Bommeli Diagnostic) .

$$INsample = ODneg - ODsample$$

Analyze the samples in relation to the negative and the positive controls with the formula:

$$\text{Value (\%)} = \frac{ODneg - OD\ sample}{ODneg - ODpos} \times 100\ \%$$

Table 2 Interpretation

Value	< 40 %	40-50%	>50%
Interpretation	Negative	ambiguous	positive

(Bommeli Diagnostic)

RESULTS

1. Cloning of cassette for the expression of E2 gene

1.1 Construction of E2 gene of CSFV and signal peptide

RNA of CSFV was extracted and used as the template for RT-PCR. The RT-PCR products of E2 gene contained approximately 1,000 bp (Figure 7).

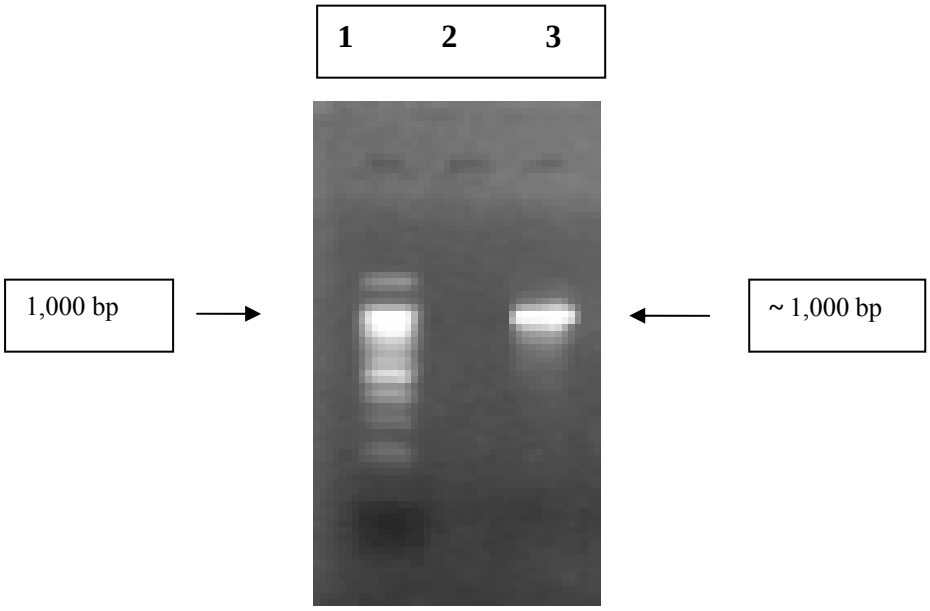


Figure 7 Analysis of PCR products of E2 gene by agarose gel electrophoresis

Lane 1 = DNA marker ; Lane 2 = negative control ;

Lane 3 = PCR products of E2 gene aproximately 1000 bp in size

In order to get TNF-α RNA, peripheral blood sample was used for RNA extraction. The RT-PCR products of signal peptide sequence of TNF-α gene showed the amplified products containing approximately 100 bp (Figure 8).

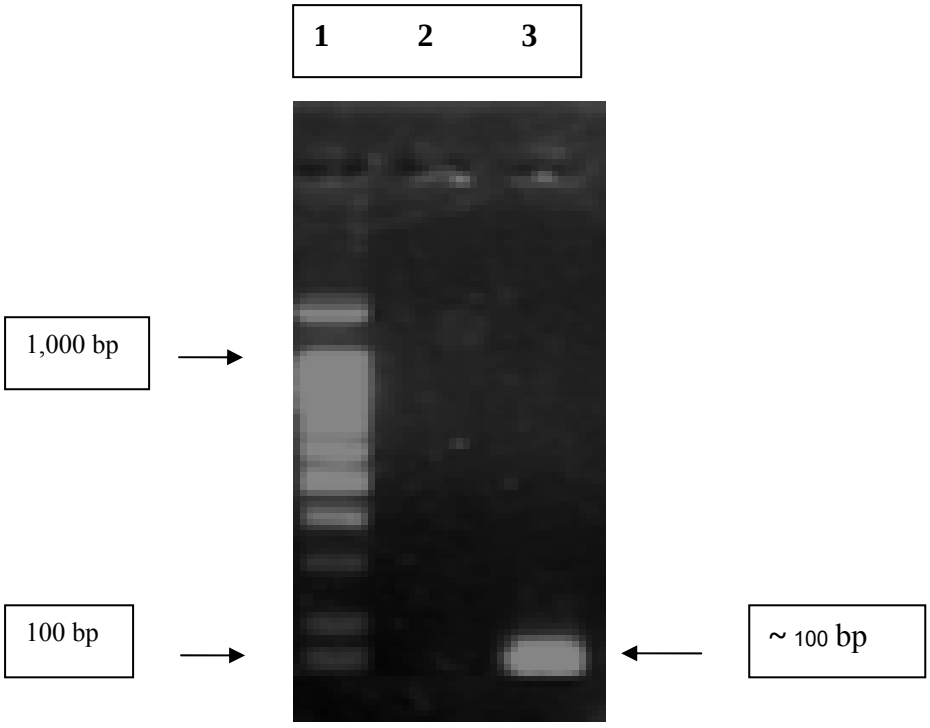


Figure 8 Analysis of PCR products of signal peptide of TNF-α gene by agarose gel electrophoresis.

Lane 1 = DNA marker ; Lane 2 = negative control ;

Lane 3 = PCR products of signal peptide of TNF-α aproximately 100 bp in size

For cloning of the cassette for the expression of E2 gene, E2 gene fragments were ligated to plasmid pcDNA 3.1. The positive colonies were primarily checked for the possession of the recombinant plasmid using PCR assay (Figure 9). The positive clone showed the PCR products approximately 1,000 bp. The restriction enzyme digestion of the selected clone *E. coli* gave E2 pcDNA 3.1 plasmid 4600 bp and E2 pcDNA 3.1 plasmid cut by two restriction enzymes showed the product size at approximately 1,000 bp (Figure 10).

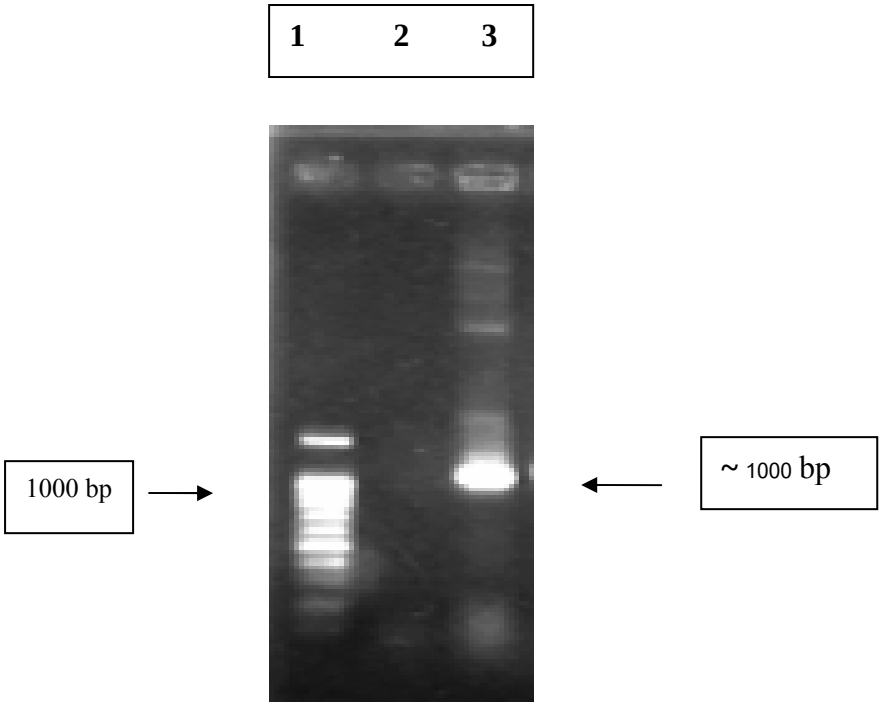


Figure 9 Agarose gel electrophoresis analysis of PCR assay of the selected clone of *E. coli* strain DH5-α.

Lane 1 = DNA marker ; Lane 2 = negative control ;

Lane 3 = positive clone showed the PCR products aproximately ~1,000 bp in size

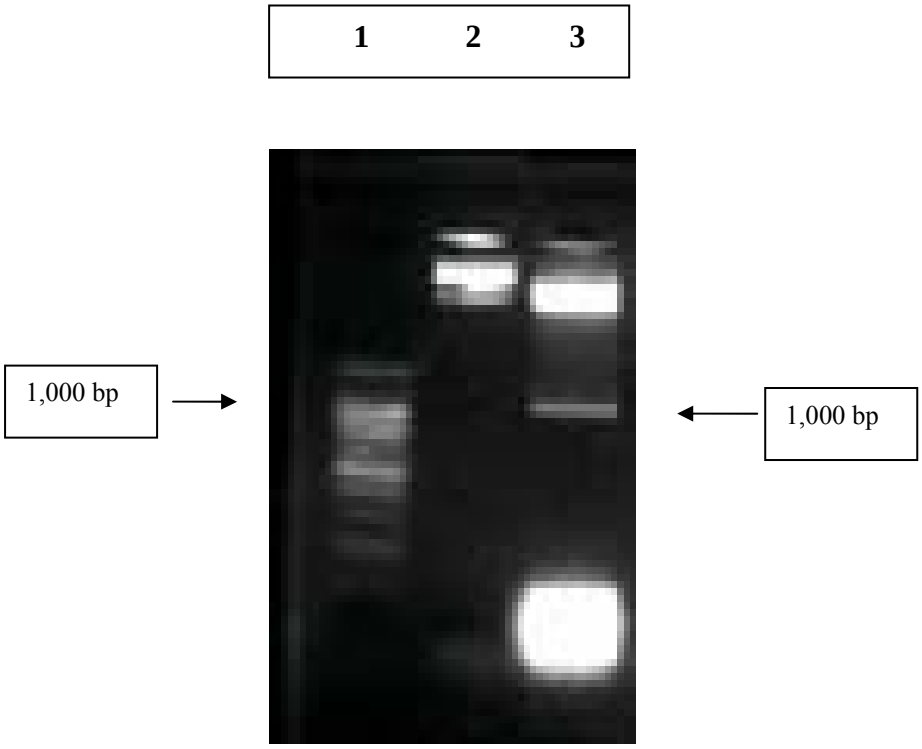


Figure 10 Agarose gel electrophoresis of restriction enzyme digestion of the selected clone of *E. coli* strain DH5-α.

Lane 1 = DNA marker ; Lane 2 = E2 pcDNA 3.1 plasmid

Lane 3 = E2 pcDNA 3.1 plasmid after cut by two restriction enzymes, *Eco*RI and *Xba*I.

Signal peptide TNF-α fragments were ligated to plasmid E2 pcDNA 3.1. The positive colonies were primarily checked for the possession of the recombinant plasmid using PCR assay (Figure 11). The positive clone showed the PCR products. of 1100 bp. The restriction enzyme digestion of the selected clone *E. coli* gave TNF-α-E2 pcDNA 3.1 plasmid 4700 bp and TNF-α-E2 pcDNA 3.1 plasmid cut by two restriction enzymes showed the product size at approximately 1,100 bp (Figure 12).

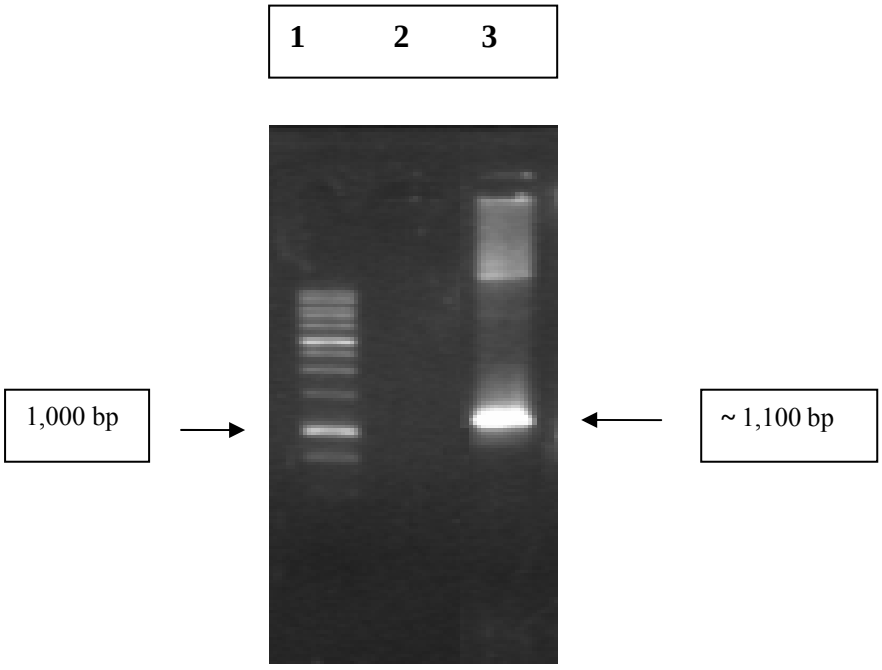


Figure 11 Agarose gel electrophoresis analysis of PCR assay of the selected clone of *E. coli* strain DH5-α.

Lane 1 = DNA marker ; Lane 2 = negative control ;

Lane 3 = positive clone showed the PCR products aproximately ~1100 bp in size

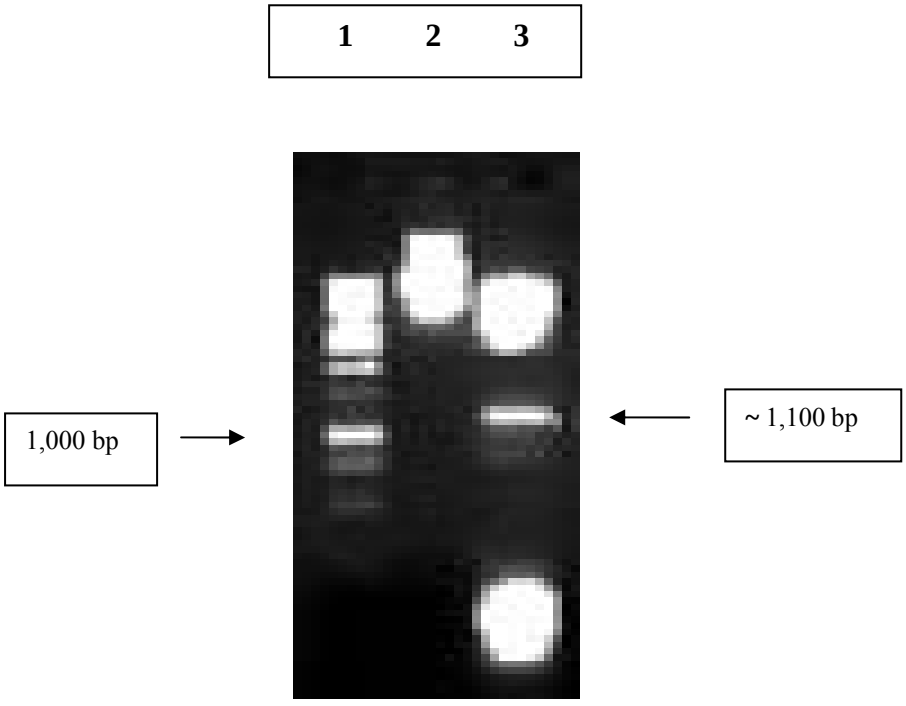


Figure 12 Agarose gel electrophoresis of restriction enzyme digestion of the selected clone of *E. coli* strain DH5-α.

Lane 1 = DNA marker; Lane 2 = TNF-α-E2 pcDNA 3.1 plasmid

Lane 3 = TNF-α-E2 pcDNA 3.1 plasmid after cut by two restriction enzymes, *Bam*HI and *Xba*I.

DNA sequencing of PCR product of E2 gene contained 1,000 bp. The sequence comparison of the E2 gene with the reference sequence of Riem and Chinese strains showed 99% nucleotide sequence homology (Figure 13). This result confirmed that the recombinant E2 have correct sequence.

```
Riems strain        ------------------------------------------------------------
Chinese strain      ------------------------------------------------------------
E2 Sequence         ATGGCCACCACGCTCTTCTGCCTACTGCACTTCGAGGTTATCGGCCCCCAGAAGGAAGAG 60

Riems strain        ------------------------------------------------CCGGCTAGCCTGC 13
Chinese strain      ------------------------------------------------CCGGCTAGCCTGC 13
E2 Sequence         TTTCCAGCTGGCCCCTTGAGCATCAACCCTCTGGCCCAAGGAGAATTCCGGCTAGCCTGC 120
                                                                    ************

Riems strain        AAGGAAGATTACAGGTACGCAATATCGTCAACCGATGAGATAGGGCTACTTGGGGCCGGA 73
Chinese strain      AAGGAAGATTACAGGTACGCAATATCGTCAACCGATGAGATAGGGCTACTTGGGGCCGGA 73
E2 sequence         AAGGAAGATTACAGGTACGCAATATCGTCAACCGATGAGATAGGGCTACTTGGGGCCGGA 180
                    ************************************************************

Riems strain        GGTCTCACCACCACCTGGAAGGAATACAACCACGATTTGCAACTGAATGACGGGACCGTT 133
Chinese strain      GGTCTCACCACCACCTGGAAGGAATACAACCACGATTTGCAACTGAATGACGGGACCGTT 133
E2 sequence         GGTCTCACCACCACCTGGAAGGAATACAACCACGATTTGCAACTGAATGACGGGACCGTT 240
                    ************************************************************

Riems strain        AAGGCCAGTTGCGTGGCAGGTTCCTTTAAAGTCACAGCACTTAATGTGGTCAGTAGGAGG 193
Chinese strain      AAGGCCAGTTGCGTGGCAGGTTCCTTTAAAGTCACAGCACTTAATGTGGTCAGTAGGAGG 193
E2 sequence         AAGGCCAGTTGCGTGGCAGGTTCCTTTAAAGTCACAGCACTTAATGTGGTCAGTAGGAGG 300
                    ************************************************************

Riems strain        TATTTGGCATCATTGCATAAGAAGGCTTTACCCACTTCCGTGACATTCGAGCTCCTGTTC 253
Chinese strain      TATTTGGCATCATTGCATAAGAAGGCTTTACCCACTTCCGTGACATTCGAGCTCCTGTTC 253
E2 sequence         TATTTGGCATCATTGCATAAGAAGGCTTTACCCACTTCCGTGACATTCGAGCTCCTGTTC 360
                    ************************************************************

Riems strain        GACGGGACCAACCCATCAACTGAGGAAATGGGAGATGACTTCAGGTCCGGGCTGTGCCCG 313
Chinese strain      GACGGGACCAACCCATCAACTGAGGAAATGGGAGATGACTTCAGGTCCGGGCTGTGCCCG 313
E2 sequence         GACGGGACCAACCCATCAACTGAGGGAATGGGAGATGACTTCAGGTTCGGGCTGTGCCCG 420
                    ************************ ******************* *************

Riems strain        TTTGATACGAGTCCTGTTGTTAAGGGAAAGTACAATACGACCTTGTTGAACGGTAGTGCT 373
Chinese strain      TTTGATACGAGTCCTGTTGTTAAGGGAAAGTACAATACGACCTTGTTGAACGGTAGTGCT 373
E2 sequence         TTTGATACGAGTCCTGTTGTTAAGGGAAAGTACAATACGACCTTGTTGAACGGTAGTGCT 480
                    ************************************************************
```

Figure 13 Alignment of TNF-α-E2 pcDNA3.1 plasmid (E2 sequence) comparing to E2 gene of CSFV Riems strain and Chinese strains from GenBank using DNASIS and ClustalW program. * = identical nucleotides found in TNF-α-E2 pcDNA3.1 plasmid and E2 gene of both strains from GenBank

```
Riems strain        TTCTATCTTGTCTGCCCAATAGGGTGGACGGGTGTCATAGAGTGCACAGCAGTGAGCCCA 433
Chinese strain      TTCTATCTTGTCTGCCCAATAGGGTGGACGGGTGTCATAGAGTGCACAGCAGTGAGCCCA 433
E2 sequence         TTCTATCTTGTCTGCCCAATAGGGTGGACGGGTGTCATAGAGTGCACAGCAGTGAGCCCA 540
                    ************************************************************

Riems strain        ACGACTCTGAGAACAGAAGTGGTAAAGACCTTCAGGAGAGACAAGCCCTTTCCGCACAGA 493
Chinese strain      ACAACTCTGAGAACAGAAGTGGTAAAGACCTTCAGGAGAGACAAGCCCTTTCCGCACAGA 493
E2 sequence         ACAACTCTGAAAACAGAAGTGGTAAAGACCTTCAGGAGAGACAAGCCCTTTCCGCACAGA 600
                    ** ******* ************************************************

Riems strain        ATGGATTGTGTGACCACCACAGTGGAAAATGAAGATTTATTCTATTGTAAGTTGGGGGGC 553
Chinese strain      ATGGATTGTGTGACCACCACAGTGGAAAATGAAGATTTATTCTATTGTAAGTTGGGGGGC 553
E2 sequence         ATGGATTGTGTGACCACCACAGTGGAAAATGAAGATTTATTCTATTGTAAGTTGGGGGGC 660
                    ************************************************************

Riems strain        AACTGGACATGTGTGAAAGGCGAGCCAGTGGTCTACACAGGGGGGCTAGTAAAACAATGT 613
Chinese strain      AACTGGACATGTGTGAAAGGCGAGCCAGTGGTCTACACAGGGGGGCTAGTAAAACAATGT 613
E2 sequence         AACTGGACATGTGTGAAAGGCGAGCCAGTGGTCTACACAGGGGGGCTAGTAAAACAATGT 720
                    ************************************************************

Riems strain        AGATGGTGTGGCTTCGACTTCGATGGGCCTGACGGACTCCCGCATTACCCCATAGGTAAG 673
Chinese strain      AGATGGTGTGGCTTCGACTTCGATGGGCCTGACGGACTCCCGCATTACCCCATAGGTAAG 673
E2 sequence         AGATGGTGTGGCTTCGACTTCGATGGGCCTGACGGACTCCCGCATTACCCCATAGGTAAG 780
                    ************************************************************

Riems strain        TGCATTTTGGCAAATGAGACAGGTTACAGAATAGTAGATTCAACGGACTGTAACAGAGAT 733
Chinese strain      TGCATTTTGGCAAATGAGACAGGTTACAGAATAGTAGATTCAACGGACTGTAACAGAGAT 733
E2 sequence         TGCATTTTGGCAAATGAGACAGGTTACAGAATAGTAGATTCAACGGACTGTAACAGAGAT 840
                    ************************************************************

Riems strain        GGCGTTGTAATCAGCACAGAGGGGAGTCATGAGTGCTTGATCGGTAACACGACTGTCAAG 793
Chinese strain      GGCGTTGTAATCAGCACAGAGGGGAGTCATGAGTGCTTGATCGGTAACACGACTGTCAAG 793
E2 sequence         GGCGTTGTAATCAGCACAGAGGGGAGTCATGAGTGCTTGATCGGTAACACGACTGTCAAG 900
                    ************************************************************

Riems strain        GTGCATGCATCAGATGAAAGACTGGGCCCTATGCCATGCAGACCTAAAGAGATTGTCTCT 853
Chinese strain      GTGCATGCATCAGATGAAAGATTGGGCCCTATGCCATGCAGACCTAAAGAGATTGTCTCT 853
E2 sequence         GTGCATGCATCAGATGAAAGACTGGGCCCTATGCCATGCAGACCTAAAGAGATTGTCTCT 960
                    ******************** ***************************************

Riems strain        AGTGCTGGACCTGTAAAGAAAACCTCCTGTACATTCAACTACACAAAAACTTTGAAGAAC 913
Chinese strain      AGTGCTGGACCTGTAAAGAAAACCTCCTGTACATTCAACTACACAAAAACTTTGAAGAAC 913
E2 sequence         AGTGCTGGACCTGTAATGAAAACCTCCTGTACATTCAACTACACAAAAACTTTGAAGAAC 1020
                    *************** ******************************************

Riems strain        AGGTACTATGAGCCCAGGGACAGCTACTTCCAGCAATATATGCTTAAGGGTGAGTATCAG 973
Chinese strain      AGGTACTATGAGCCCAGGGACAGCTACTTCCAGCAATATATGCTTAAGGGTGAGTATCAG 973
E2 sequence         AGGTACTATGAGCCCAGGGACAGCTACTTCCAGCAATATATGCTTAAGGGTGAGTATCAG 1080
                    ************************************************************

Riems strain        TACTGGT-------- 980
Chinese strain      TACTGGT-------- 980
E2 sequence         TACTGGTAGTCTAGA 1095
                    *******
```

Figure 13 (Continued)

1.2 Construction of E2 pBAD/D-TOPO plasmids

The PCR products of E2 gene was amplified from TNF-α-E2 pcDNA 3.1 plasmid. The result showed the amplified products approximately 1,000 bp as shown in Figure 14.

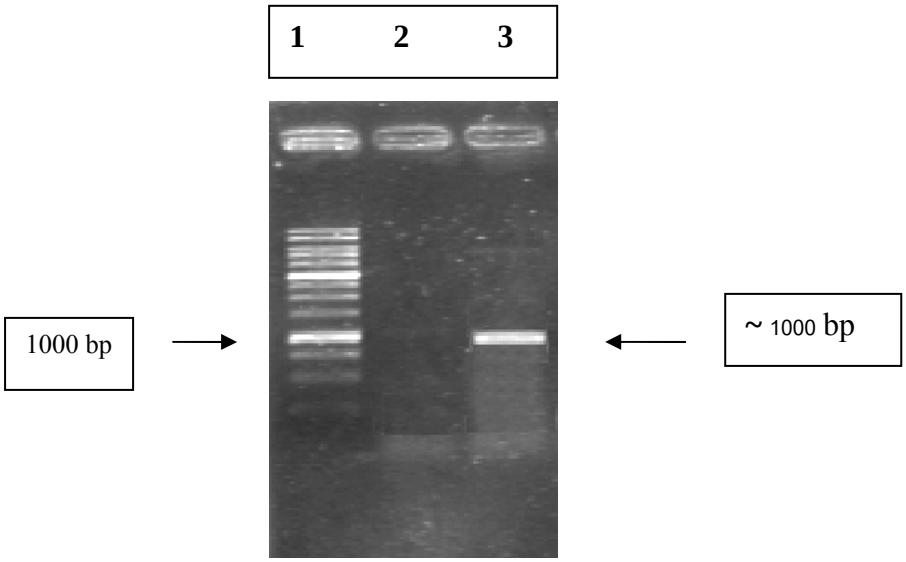


Figure 14 Analysis of PCR products of E2 gene by agarose gel electrophoresis

Lane 1 = DNA marker ; Lane 2 = negative control ;

Lane 3 = PCR product of E2 has aproximately 1000 bp in size

The E2 pBAD/D-TOPO plasmids were used to transform *E. coli* strain TOP10. The transformant was primarily checked for the possession of the recombinant plasmid using PCR assay. The result showed that the clone has possessed the gene encoding for E2 gene which was approximately 1,000 bp in length (Figure 15)

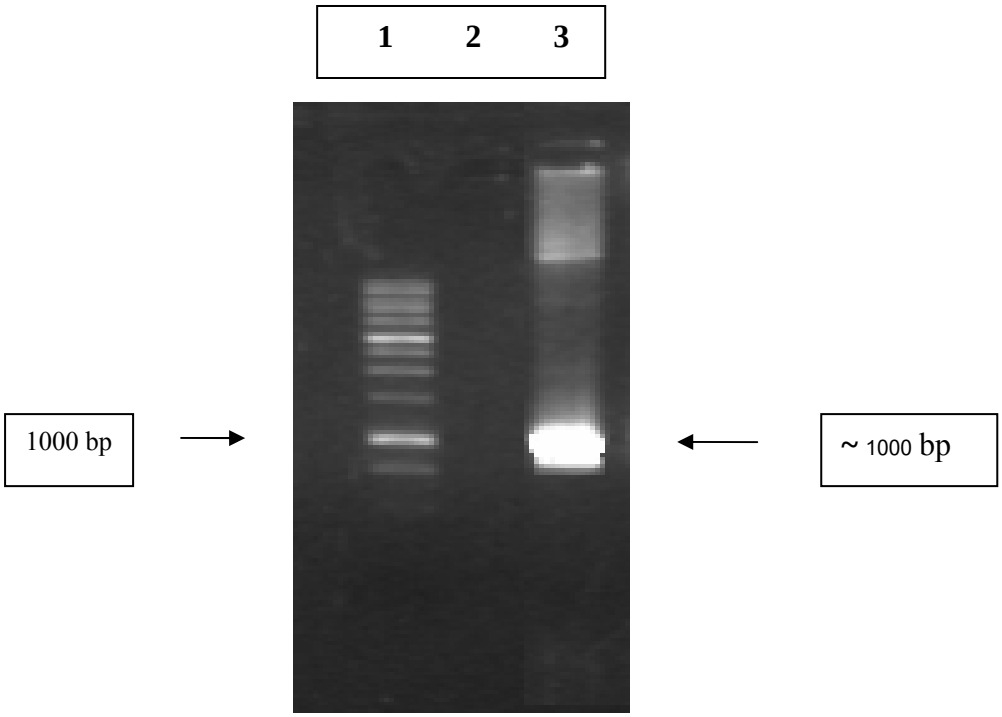


Figure 15 Agarose gel electrophoresis analysis using PCR assay of the selected clone of *E. coli* strain Top 10.

Lane 1 = DNA marker; Lane 2 = negative control ;

Lane 3 = positive clone showed the PCR products aproximately ~1000 bp in size

2. Expression of the recombinant E2 protein

2.1 Concentration analysis of protein expression

The different concentrations of arabinose in culture medium were tested for the induction ability of recombinant E2 protein expression in *E. coli*. The presence of recombinant E2 protein was shown as a band of 52 kDa. Addition of arabinose at concentrations of 0.2% to 0.00002% was able to induced expression of recombinant E2 protein (Figure 16) whereas the extract of crude cells without arabinose showed not induced expression of recombinant E2 protein. The optimal concentration for the expression of recombinant protein was shown to be 0.002% final concentration of arabinose.

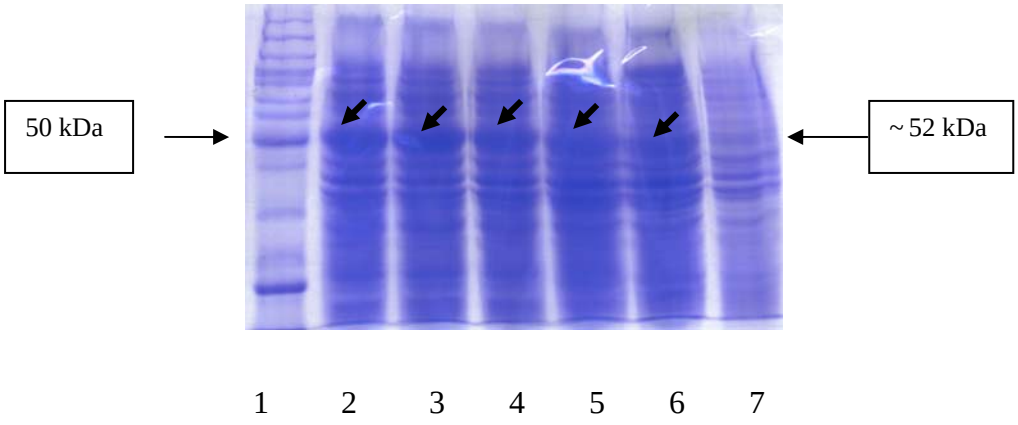


Figure 16 Concentration analysis of arabinose for the recombinant E2 protein expression using *E. coli* strain Top 10 containing E2 pBAD/D-TOPO plasmid by 12% SDS-PAGE.

Lane 1 = protein marker;

Lane 2 = induction with $2x10^{-1}$ % final concentration of arabinose;

Lane 3 = induction with $2x10^{-2}$ % final concentration of arabinose;

Lane 4 = induction with $2x10^{-3}$ % final concentration of arabinose;

Lane 5 = induction with $2x10^{-4}$ % final concentration of arabinose;

Lane 6= induction with $2x10^{-5}$ % final concentration of arabinose;

Lane 7 = crude cells before induction.

2.2 Time-Course analysis of protein expression

The analysis of the expression of recombinant E2 protein after induction at different time points was conducted. The E2 protein expression was shown as a band of 52 kDa. *E. coli* was incubated with arabinose at concentration of 0.002% for 2,4,6 or 8 hours. The E2 protein was initially found at 2 hour induction. The protein levels increased at 4 and 6 hours incubation. The bands of protein of *E. coli* after incubation with arabinose at 6 and 8 hours were identical. The optimal induction period of the expression of recombinant protein was 6 hours (Figure 17).

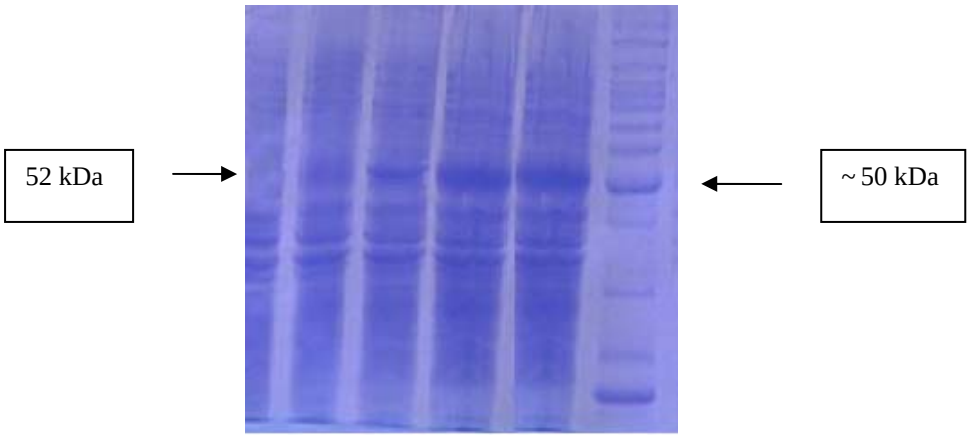


Figure17 Time course analysis of protein expression. The results show both non-induced *E. coli* strain Top 10 and *E. coli* strain Top 10 containing E2 pBAD/D-TOPO plasmid by 12% SDS-PAGE.

Lane 1 = crude cells before induction;

Lane 2 = after induction 2 hours;

Lane 3 = after induction 4 hours;

Lane 4 = after induction 6 hours;

Lane 5 = after induction 8 hours;

Lane 6 = protein marker.

2.3 Standard *E. coli* expression culture

The condition of *E. coli* strain Top 10 containing E2 pBAD/D-TOPO plasmid was 0.2 % arabinose (0.002% final concentration) and 6 hours incubation as shown in Figure 18.

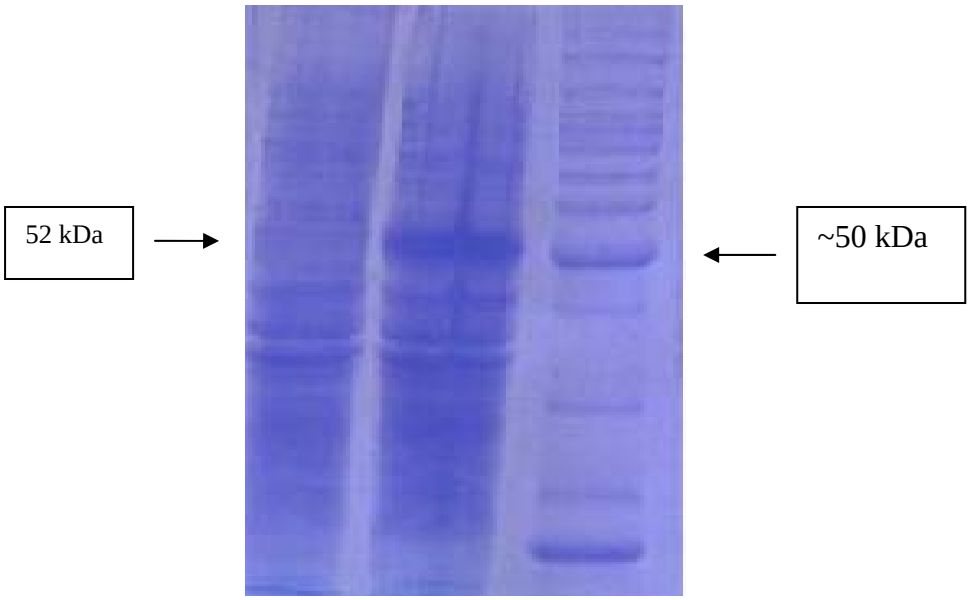
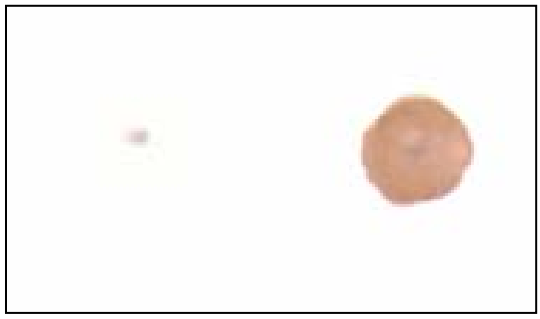


Figure18 12% SDS-PAGE analysis of recombinant E2 protein expression in Top 10 cells induced 0.2 % arabinose (0.002% final concentration) at 6 hours incubation. Total lysates were electrophoresed in 12% SDS-PAGE.

Lane 1 = non-induced Top 10 cells; Lane2 = induced cells lysates; Lane 3= protein marker .

2.4 Determination of crude recombinant protein by Dot blot analysis

The presence of the recombinant E2 protein was tested with mouse anti-E2 monoclonal antibody using dot blot analysis. The recombinant E2 protein was clearly reacted with the mouse anti-E2 monoclonal antibody. Positive reaction was shown as dark brown color spot. The extract from *E. coli* Top 10 cells containing E2 pBAD/D-TOPO plasmid was stained brown when the protein extract from normal *E. coli* TOP10 cells was negative (Figure 19).



1 2

Figure 19 Dot blotting analysis of crude lysates from normal *E. coli* TOP10 cells and *E. coli* TOP10 cells containing E2 pBAD/D-TOPO plasmid. Result showed the recombinant E2 protein (2) clearly react with the anti-E2 monoclonal antibody but not in the normal cell lysates (1).

2.5 Determination of crude recombinant protein expression by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The result from the western blotting showed the immunological reaction of anti-E2 monoclonal antibody to the crude recombinant protein E2 as the band of 52 kDa, but not to the protein extract from normal *E. coli* Top10 cells (Figure 20).

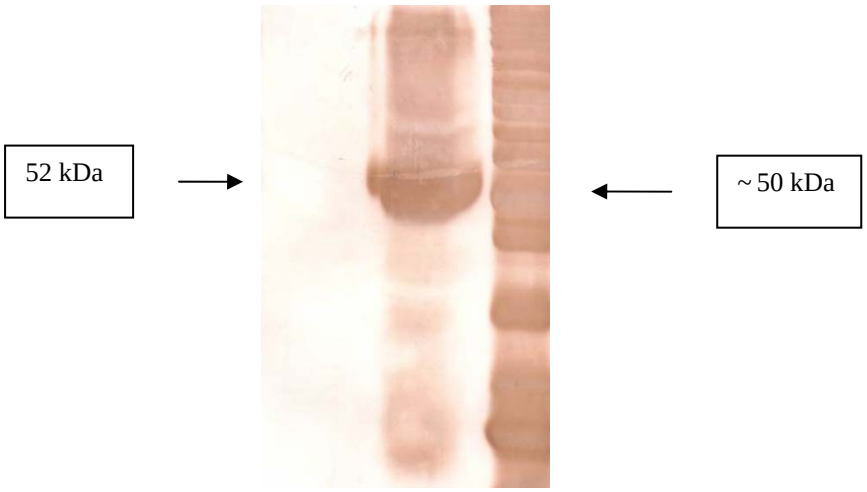


Figure 20 Western blotting analysis of the immunological reaction between the crude lysates of recombinant protein E2 and the protein extract from normal *E. coli* Top10 cells with anti E2 monoclonal antibody.

Lane1 = protein extract from normal *E. coli* Top10 cells;

Lane2 = recombinant protein E2;

Lane3 = protein marker.

3. Purification of the Recombinant Protein Using Ni-NTA

3.1 The purification of 6x His-tagged proteins from *E. coli* under denaturing condition

The recombinant E2 protein was purified under denaturing condition using the Ni-NTA agarose and was analyzed in SDS-PAGE (Figure 21). During the purification step, endogenous proteins with histidine residues that interact with the Ni-NTA groups can be washed out of the matrix with stringent conditions achieved by lowering the pH to 6.3 (buffer C). Monomers generally elute at approximately pH 5.9 (buffer D), whereas aggregates and proteins that contain more than one 6xHis tag elute at approximately pH 4.5 (buffer E).

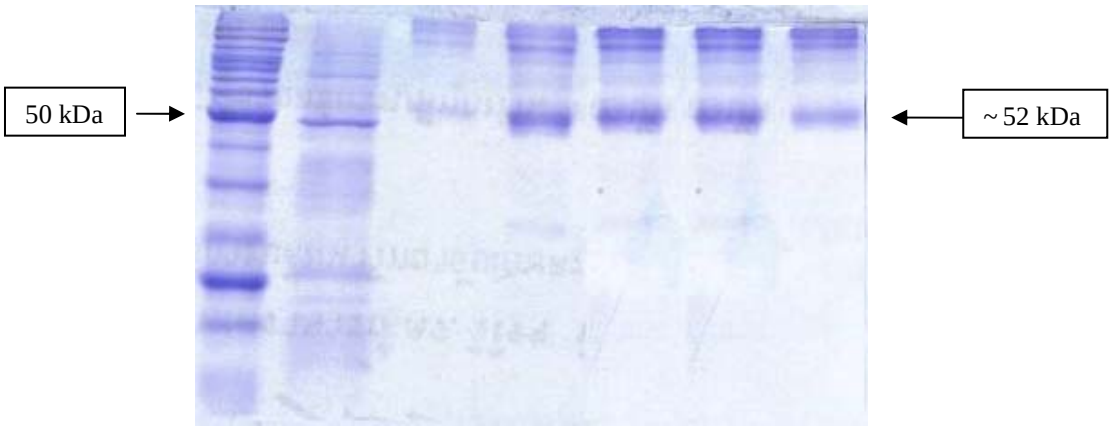


Figure 21 12% SDS-PAGE showed the purified protein present in each fraction of the purification step of *E. coli* strain Top10 containing E2 pBAD/D-TOPO plasmid .

Lane 1 = protein marker; Lane 2 = eluted fraction after wash the resin with washing buffer C (pH 6.3); Lane 3 = eluted fraction from eluted buffer D (pH 5.9); lane 4-7 = eluted fraction from eluted buffer E (pH 4.5)

3.2 Determination of the recombinant protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The western blotting results showed the immunological reaction of anti-E2 monoclonal antibody to the purified recombinant protein E2, but not to the protein purified from *E. coli* strain Top10 without E2 pBAD/D-TOPO plasmid (Figure 22).

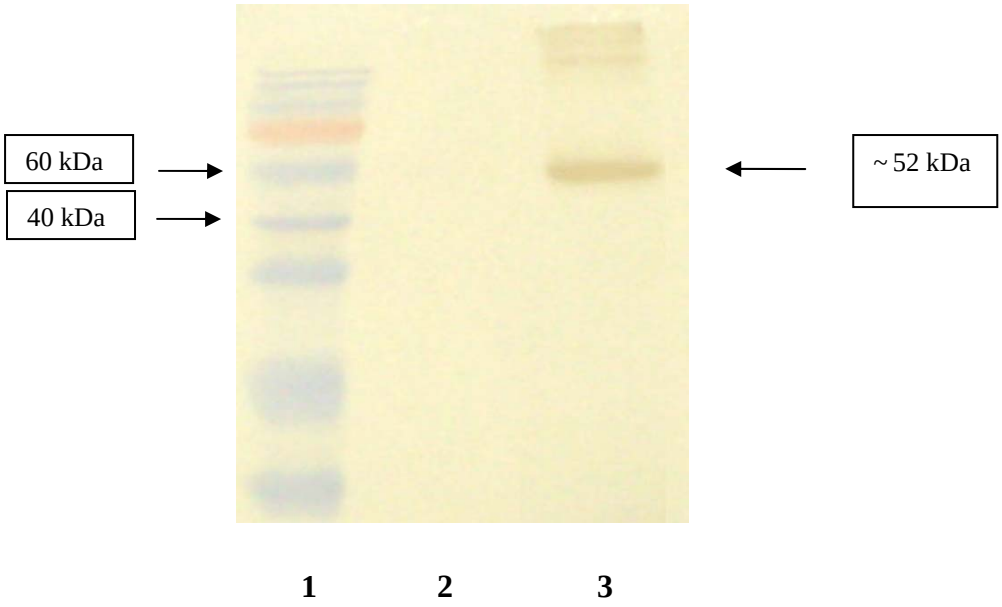


Figure 22 Western blotting analysis of the immunological reaction between the purified recombinant E2 protein and the protein purified from normal *E. coli* strain TOP10 with the anti- E2 monoclonal antibody.

Lane1 = Protein marker;

Lane2 = Protein purified from *E. coli* without pBADE2 plasmid;

Lane3 = Purified recombinant protein E2.

4. Antibody detection against the E2 protein in rabbit by CHEKIT-CSF-SERO enzyme immunoassay (EIA) kit after immunization with recombinant E2 proteins

The CHEKIT-CSF-SERO enzyme immunoassay (EIA) kit was used to the detection of antibodies against the recombinant E2 protein of CSFV in rabbits' serum. All six rabbits injected with three doses (400 µg/dose) of the recombinant protein E2 developed % value more than 50% within 6 weeks. However, all three rabbits with the protein purified from *E. coli* without E2 pBAD/D-TOPO plasmid developed % value lower than 10% in 6 weeks. The mean of % value of control group in week 0, 2, 4, 5 and 6 was 5.67%, 3.67%, 5.00%, 5.67% and 5.00% respectively. The levels of Ab against E2 protein of control group was not increased within 6 weeks. The relation between % value and week post-injection was shown in figure 23.

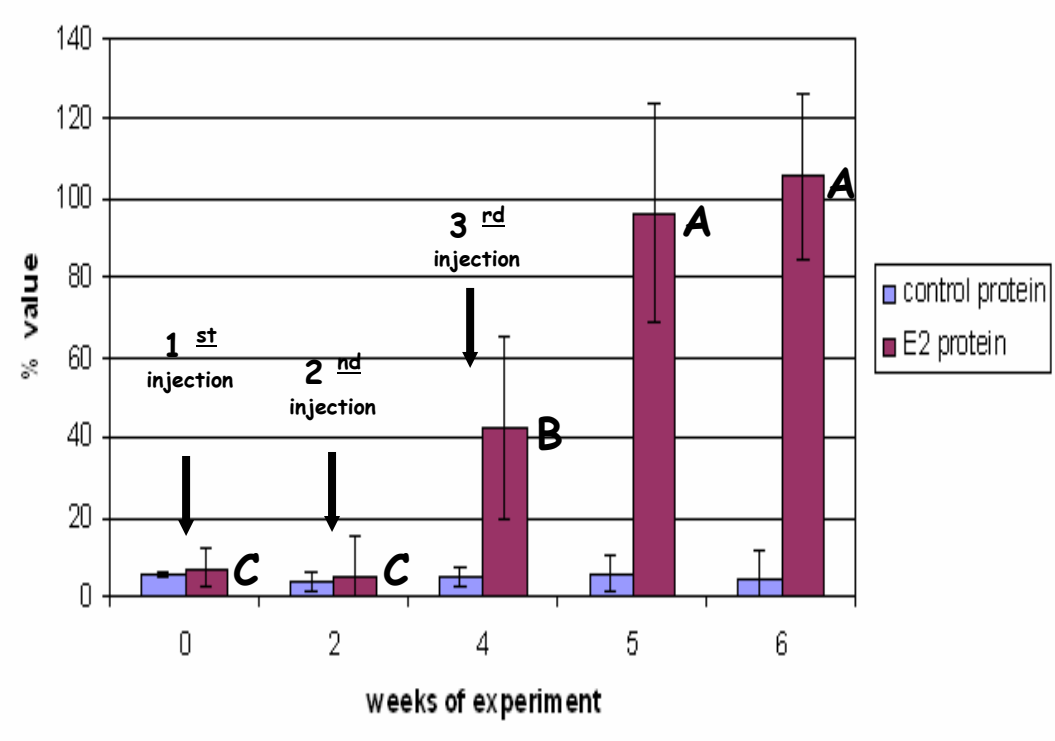


Figure 23 The relation between % value and weeks post-injection of six Rabbits injected with the recombinant E2 protein and three rabbits injected with purified protein from *E. coli* without E2 pBAD/D-TOPO plasmid (control). Difference letter (A, B and C) showed statistically significant difference ($p < 0.01$) mean of % value in treatment group.

Table 3 The comparation of % value (mean±SD) between rabbits injected with purified protein of Top 10 without pBADE2 (control) and rabbits injected with recombinant E2 protein (treatment)

	% value (mean ± S.D.)				
	week 0	wee k2	week 4	week 5	week 6
Control	5.67±0.58	3.67±2.52	5.00±2.65	5.67±4.62	5.00±7.00
Treatment	7.17±4.95	5.83±10.13	42.50±22.81*	96.33±27.66*	105.80±20.90*

***** Statistically significant higher values ($p<0.01$) between control and treatment Groups at each time point

The result showed treatment group developed significantly higher mean of % value than control group in week 4,5 and 6 ($p<0.01$) (Table 3). After injected rabbits with two doses (400 µg/dose) of recombinant E2 protein can stimulated the antibody againat E2 protein in rabbits'serum. The mean of % value of treatment group between week 0 and week 2 was not significantly different but between week 2 and week 4 increased 7 fold. The mean of % value at week 5 increased 2 fold from week 4. The mean of % value between week5 and week 6 was not significantly different, although peaked at week 6.

DISCUSSION

Cloning of E2 gene

In this study, the recombinant E2 gene was successfully cloned and expressed. The restriction endonuclease assay and the PCR assay were conducted to confirm the correctness of the gene encoding for the E2 protein and the result showed 1000 bp fragment as analyzed by the DNASIS program. Afterwards, the recombinant genes were sequenced and compared with the reported E2 gene (GenBank). The results showed nucleotide sequences of the recombinant plasmid have 99% homology with the E2 gene of Chinese strain and Riems strain reported in the GenBank. This result confirmed that the clone recombinant E2 have correct sequences. Comparison of amino acid sequences of the recombinant E2 with the reported E2 protein in GenBank showed 99% homology. This result confirmed that the cloned E2 gene was successfully translated to the recombinant E2 protein in the *E. coli* expression system.

The DNA fragments encoding the protein E2 with and without c-terminal transmembrane region (TMR) were reported to possess approximately 1,126 bp and 1,020 bp respectively (Hulst *et al.*, 1993).

The E2 gene of the viral genome after removed the gene encoding TMR contained approximately 1025 bp (Markowska-Daniel *et al.*, 2001)

In this study, the E2 gene without TMR showed approximately 1000 bp fragment. The result showed the size of PCR product nearly the previously research.

The recombinant E2 expression system

The expression of CSF E2 protein in insect (Hulst *et al.*, 1993) and mammalian cells (Yu *et al.*, 2001) has been reported. However, the expression efficiency and purity of recombinant products by insect and mammalian cell systems was not sufficient for develop subunit vaccine (Kesik *et al.*, 2004).

The baculovirus–insect cell expression system is versatile and widely used for the high-level production of heterologous (eukaryotic) proteins (Possee, 1997). The proteins are often properly folded and post-translationally modified to obtain similar biological activities to their authentic counterparts (Vialard *et al.*, 1995). However, for both classical and bacmid recombinants, the rapid generation and accumulation of defective interfering viruses (DIs) upon passage in infected cells is still a major obstacle for the efficient large-scale production of virus and heterologous proteins in cell culture systems (Kool *et al.*, 1991; Wickham *et al.*, 1991) since repeated baculovirus infections in cultured insect cells lead to the generation of defective interfering viruses (DIs), which accumulate at the expense of the intact helper virus and compromise heterologous protein expression.. This phenomenon therefore causes a sharp drop in protein production upon serial passage of baculoviruses in cultured insect cells (Krell, 1996).

Escherichia coli (*E. coli*) facilitates protein expression by its relative simplicity, its inexpensive and fast high-density cultivation, the well-known genetics and the large number of compatible tools available for biotechnology. However, when expressing in *E. coli* systems many recombinant proteins become insoluble and are trapped in inclusion bodies. Another drawback is that proteins expressed in bacteria will not have any eukaryotic post-translational modifications (Sorensen and Mortensen, 2005).

E. coli produces proteins without modifications, such as glycosylation. This is particularly useful for proteins for which glycosylation is not a requirement, but which could be a problem if the protein is produced in other systems (e.g. yeast), which can over-glycosylate, or add inappropriate carbohydrates to the protein, which could lead to reduced or no activity of the expressed protein and potentially create a risk of immunogenicity (Pissarra, 2000).

In this research, *E. coli* expression system was chosen to express recombinant protein E2 because this system has been well developed and proposed to be highly efficiency in producing the recombinant protein. The pBAD Directional TOPO expression kit (Invitrogen) was chosen for the expression of recombinant protein E2 in *E. coli*. The pBAD/D-TOPO vector carring the *ara*BAD promoter which has dose-dependent regulation of protein expression with L-arabinose, a C-terminal V5-His tag for convenient purification and detection of fusion protein and the kanamycin resistance gene for selection of transformed *E. coli* and fusion to thioredoxin which can increase translation efficiency and, in some cases, solubility of eukaryotic

proteins expressed in *E. coli*. The expression of the E2 fusion protein which contains a tagged molecule with thioredoxin and six histidines at its N-terminus offers an alternative fast and efficient way to obtain sufficient quantities of purified product. Recombinant E2 protein carrying six consecutive histidine residues on the N-terminus can be purified using a resin containing nickel ions (Ni^{2+}) that have been immobilized by covalently attached nitrilotriacetic acid.

Regulation of the arabinose operon in *E. coli* is directed by the product of the *ara*C gene. In the absence of arabinose, the AraC dimer contacts the O2 site located within the *ara*C gene, 210 base pairs up-stream from the *araBAD* promoter. The other half of the AraC dimer contacts the O1 site in the promoter region and a DNA loop is formed. Transcription from the *araBAD* promoter and the *araC* promoter is repressed by the AraC loop conformation. Upon binding of arabinose the AeaC dimer changes its conformation, binding to the O2 site is replaced by binding to the I site at the *araBAD* promoter and transcription by RNA polymerase initiates (Appendix III).

In previous study, expression of GFP was induced by the addition of varying concentrations of L-arabinose. The result showed an arabinose concentration of 0.2% for maximal fluorescence yields (Morgan-kiss *et al.*, 2002). In this research, optimal concentration of arabinose for induction E2 protein was 0.2% as same as the concentration of the previous report.

Expression of E2 protein in *E. coli*

Using the western blot analysis the E2 protein expression in *E. coli* was shown as a band of 52 kDa, which including 3 kDa of 6xhis and 13 kDa of thioredoxin from vector. Dot blotting was used to determine structural correctness of the recombinant protein which has advantages of less time and Ab consumption however the dot blotting can not give size of the recombinant protein whereas western blot analysis can provide information about the size of the protein. The recombinant E2 protein was shown to have specific interactions with monoclonal IgG antibody against E2 protein in both assays. This result indicated the recombinant E2 protein possesses the correct structure.

Recombinant E2 protein with its TMR expressed in virus Bac E2 [+] was similar in size (51 to 54 kDa) to the size of E2 expressed in swine kidney cells infected with CSFV. In contrast, recombinant virus BacE2 [-], which expressed E2 without a TMR, generated a protein that was secreted from the cells. The fraction of this protein had a sightly lower molecular mass (49 to 52 kDa) than wild-type (Hulst *et al.*, 1993).

A fragment of non-glycosylated E2 antigen of CSFV, lacking the trans-membrane anchor (TM^-) of the native glycoprotein (1,058 bp), was produced in recombinant *E. coli* strain BL21 (DE3). The E2 protein expression was shown as a band of 38 kDa (Kesik *et al.*, 2004).

In this study, the molecular weight of E2 protein was similar in size to the size of E2 protein produced in *E. coli* system (Kesik *et al.*, 2004) but different to the size of E2 expressed in eukaryote system (Hulst *et al.*, 1993). The result suggested that the E2 protein produced in *E. coli* not have any eukaryotic post-translational modifications.

Immunogenicity of recombinant E2 protein

It has previously been demonstrated that the E2 protein, stimulates virus neutralizing antibody and can protect pigs when expressed from a recombinant vaccinia virus (Konig *et al.*, 1995), a recombinant pseudorabies virus (Peeters *et al.*, 1997), given as baculovirus expressed protein (Bouma *et al.*, 1999), a DNA vaccine (Yu *et al.*, 2001) and inclusion bodies from recombinant bacteria (Kesik *et al.*, 2004).

The convincing evidence for the antigenic integrity of E2 was obtained from the vaccination experiments with rabbits. Control group, three rabbits, was injected with purified Top10 without pBADE2 and treatment group, six rabbits, was injected with three doses of 400 µg recombinant E2 protein. Rabbits' serum was detected E2 antibody by CSF-SERO kit (Bommeli Diagnostics). The E2 protein of CSFV produced in recombinant *E. coli* was shown to induce anti-E2 specific antibodies in serum of six rabbits of the treatment group but not in three control rabbits. The result showed treatment group developed significant ($p<0.01$) mean of % value higher than control group.

E2 protein has been shown its ability to protect pigs challenged with 50% lethal dose of CSFV strain Brescia, by inducing high titer of neutralizing antibodies against CSFV. It indicates that E2 expressed in insect cells is an excellent candidate for develop of a new, safe, and effective CSFV subunit vaccine (Hulst *et al.*, 1993).

In this experiment the recombinant E2 protein expressed in *E. coli* has ability to stimulate significant amount of antibody production in rabbits. The levels of Ab against E2 protein increased more than 5 fold at week 4 of post-injection and peaked at week 6.

The results suggested that the E2 protein may play important role in inducing protective activity against CSFV infection and promising as a safe, effective, and differentiable CSFV subunit vaccine. Additional experiments in CSFV-free pigs are required to establish conclusions for using protein E2 as subunit vaccine.

CONCLUSION

In this experiment, the E2 gene was successfully cloned and expressed in pBAD directional TOPO expression system. The recombinant E2 protein had molecular mass approximately of 52 kDa. The recombinant E2 protein was clearly reacted with the mouse anti-E2 antibody by dot and western blot analyses. Rabbits injected with the recombinant E2 protein had the antibody response against CSFV as shown by CHEKIT-CSF-SERO enzyme immunoassay (EIA) but the rabbits injected with protein normal *E. coli* strain TOP10 not. The recombinant E2 protein was clearly shown the ability to stimulate the strong immune response against E2 protein of CSFV. The E2 protein had been shown to play important role in inducing protective activity against CSFV infection and promising as a safe, effective, and differentiable CSFV subunit vaccine. The E2 protein has been found to be the main envelope glycoprotein capable of inducing immunity to infection (Muyldermans, 1993; van Rijn, 1994). However, the recombinant E2 protein in this study was produced by *E. coli* expression system. This result showed that the glycosylation of E2 protein might not necessary for the stimulation of the specific antibody against E2 protein of CSFV. The *E. coli* expression system of recombinant E2 protein was highly efficiency and was inexpensive in producing the recombinant E2 protein. Thus, the recombinant E2 protein might be the more appropriate vaccine for helping to the eradication of classical swine fever from Thailand.

LITERATURE CITED

Arakawa, T., D.K.X. Chong and W.H.R. Langridge. 1998. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. **Nat. Biotechnol**. 16: 292-297.

Agterberg, M., H. Adriaanse., H. lankhof., R. Meloen and J. Tommassen. 1990. Outer membrane PhoE protein of *E. coli* as a carrier for foreign antigenic determinants: immunogenicity of epitopes of foot-and-mouth disease virus. **Vaccine**. 8: 85-91.

Anonymous, 1994. **report from the scientific veterinary committee on guidelines for a classical swine fever emergency vaccination programme.** Document VI/7389/94-EN, Commission of the European Communities.

_____, 2001. Commission Decision, **Diagnostic Manual for Classical Swine fever**. Brussels: commission of the European Community, SANco/2507/2001 rev1. Commission of the European Community, Brussels.

Biagetti, M. Greiser-Wilke. I and D. Rutili. 2001. Molecular epidemiology of classical swine fever in Italy. **Vet Microbiol**. 83: 205-215.

Billman-Jacobe, H. 1996. Expression in bacteria other than *Escherichia coli*. **Curr. Opin. Biotechnol**. 7: 500-504.

Birnboim, H.C. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. **Methods Enzymol**. 100: 243-255.

Bommeli Diagnostics. 2003. **CSF-SERO ELISA TEST KIT: Instructions**. Bommeli Diadnostics. Switzerland.

Bouma, A., A.J. de smit., E.P. de Kluijver., C. Terpstra and R.J.M., Moormaan. 1999. Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. **Vet Microbiol**. 66: 101-114.

Bouma A., J.A. Stegeman., B. Engel., E.P. de Kluijver., A.R. Elbers and M.C. De Jong. 2001. Evaluation of diagnostic tests for the detection of classical swine fever in the field without a gold standard. **J. Vet. Diagn. Invest**, 13: 383–388.

Bruschke, C.J., M.M. Hulst., R.J. Moormann., P.A. van Rijn and J.T. van Oirschot. 1997. Glycoprotein Erns of pestiviruses induces apoptosis in lymphocytes of several species**.J Virol**. 71: 6692-6696.

Cochet, O., J.L. teillaud and C. Sauts. 1998**. Immunological techniques made easy**. Chichester. Wiley.

Colijn, E.O., M. Bloemraad and G. Wensvoort. 1997. An improved ELISA for the detection of serum antibodies directed against classical swine fever virus. **Vet. Microbiol.** 59: 15–25.

Crauwels, A.P., M. Nielen., J.A. stegeman., A.R. Elbers., A.A. Dijkhuizen and M.J. Tielen. 1999. The effectiveness of routine serological surveillance: case study of the 1997 epidemic of classical swine fever in The Netherlands. **Rev Sci Tech**. 18: 627-637.

Depner, K., T. Bauer and B. Liess. 1992. Thermal and pH stability of pestiviruses. **Rev Sci Tech**. 11: 885-893.

Dewulf, J., H. Laevens., F. Koenen., K. mintiens and A. De Kruif. 2001. An E2 sub-unit marker vaccine does not prevent horizontal or vertical transmission of classical swine fever virus. **Vaccine.** 20: 86-91.

Dewulf, J., H. Laevens., F. Koenen., K. mintiens and A. De Kruif. 2001. An experimental infection with classical swine fever virus in pregnant sows: transmission of the virus, course of the disease, antibody response and effect on gestation**. J Vet Med B Infect Dis Vet Public Health**. 48: 583-591.

Dewulf, J., H. Laevens., F. Koenen., K. mintiens and A. De Kruif. 2001. Evaluation of the potential of dogs, cats and rats to spread classical swine fever virus. **Vet Rec**. 149: 212-213.

Dewulf, J., H. Laevens., F. Koenen., K. mintiens and A. De Kruif. 2000. Airborne transmission of classical swine fever virus under experimental conditions. **Vet Rec.** 147: 735-738.

Dewulf, J., H. Laevens., F. Koenen., H. vanderhallen., K. Mintiens., H., Deluyker and A. de Kruif. 2000. An experimental infection with classical swine fever in E2 sub-unit marker-vaccine vaccinated and in non-vaccinated pigs. **Vaccine.** 19 : 475-482.

Dong, X.N., K. Wei., Z.Q. Liu and Y.H. Chen. 2002. Candidate peptide vaccine induced protection against classical swine fever virus. **Vaccine.** 21: 167-173.

Dunne, H.W. 1975. **Hog cholera. In disease of swine**. 4th ed. Ed. H.W. Dunne and A.D. Leman., Ames:Iowa State Univ Press.

Dybczynski, I and A. Pluciennicak. 1988. a protocol for DNA fragment extraction from polyacrylamide gels. **Bio Techniques**. 6: 924-926.

Echelard, Y. 1996. Recombinant protein production in transgenic animals. **Curr. Opin. Biotechnol.** 7: 536-540.

Edwards, S. 2000. Survival and inactivation of classical swine fever virus. **Vet Microbiol**. 73: 175-181.

Edwards, S., A. Fukusho., P.C. Lefevre., A.,Lipowski., Z. Pejsak., P. Roehe and J. westergaard. 2000. Classical swine fever: the global situation. **Vet Microbiol.** 73: 103-119.

Elber, A.R., A. stegeman., H. Moser., H.M. Ekker., J.A. Smak and F.H. Pluimers. 1999. The classical swine fever epidemic 1997-1998 in The Netherlands: descriptive epidemiology. **Prev Vet Med.** 42: 157-184.

Fritzemeier, J., J. Teuffert., I. Greiser-Wilke., C. Staubach., H. Schluter and V. Moennig. 2000. Epidemiology of classical swine fever in Germany in the 1990s. **Vet Microbiol**. 2000. 77: 29-41.

Geisse, S., H. Gram., B. Kleuser and H.P. Kocher. 1996. Eukaryotic expression systems: a comparison. **Protein Expr. Purif.** 8: 271-282.

Gibbens, J., S. Mansley., G. Thomas., H. Morris., D. Paton., T. Drew., T. Sandvik and J. Wilesmith. 2000. Origins of the CSF outbreak.**Vet Rec**. 147: 310

Greiser-Wilke, I., J. Fritzemeier., F. Koenen., H. Vanderhallen., D. Rutili., G.M. De Mia., L. Romero., R. Rosell., J.M. Sanchez-Vizcaino and A. San. 2000. Molecular epidemiology of a large classical swine fever epidemic in the European Union in 1997-1998. **Vet Microbiol**. 2000. 77: 17-27.

Greiser-Wilke, I and V. Moennig. 2004. Vaccination against classical swine fever virus: Limitations and new strategies. **Ani Health Res Rev**. 5: 223- 226.

Haas, B., R. Ahl., R. Bohm and D. Strauch. 1995. Inactivation of viruses in liquid manure**. Rev Sci Tech**. 14: 435-445.

Hahn, J., S.h. Park., J.Y. Song., S.H. An and B.Y. Ahn. 2001. Construction of recombinant swinepox viruses and expression of the classical swine fever virus E2 protein. **J Virol Methods**. 93: 49-56.

Hammond, J.M., E.S. Jansen., C.J. Morrissy., W.V. Goff., G.C. Meehan., M.M. Williamson., C. Lenghaus., K.W. Sproat., M.E. Andrew., B.E. Coupar and M.A. Johnson. 2001a. A prime-boost vaccination strategy using naked DNA followed by recombinant porcine adenovirus protects pigs from classical swine fever. **Vet Microbiol**. 80: 101-119.

Hammond, J.M., E.S. Jansen., C.J. Morrissy., M.M. Williamson., A.L. Hodgson and M.A. Johnson. 2001b. Oral and sub-cutaneous vaccination of commercial pigs with a recombinant porcine adenovirus expressing the classical swine fever virus gp55 gene. **Arch Virol**. 146: 1787-1793.

Haq, T.A., H.S. Mason., J.D. Clements and C.J. Arntzen. 1995. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. **Science.** 268: 714-716.

Hennecken, M., J.A. Stegeman., A.R. Elbers., A. van Nes., J.A. Smak and J.H. Verheijden. 2000. Transmission of classical swine fever virus by artificial insemination during the 1997-1998 epidemic in The Netherlands: a descriptive epidemiological study. **Vet Q**. 22: 228-233.

Holmgren, A. 1985. Thioredoxin. **Ann.Rev.Biochem**. 54: 237-271.

Hulst, MM., D.F. Westra, G. Wensvoort and R.J. Moormann. 1993. Glycoprotein E1 of Hogcholera virus expressed in insect cells protects swine from hog cholera. **J. Virol**. 67: 5435-5442.

Invitrogen. 2004. **pBAD Directional TOPO Expression Kits**: Instruction manual.

Jakw, K., A.M. Ten Berge., P. Borger., G. Venema. 1995. A general method for the consecutive integration of single copies of a heterologous gene at multiple locations in the *Bacillus subtilis* chromosome by replacement recombination. **Appl Environ Microbiol**. 61:4244-4250

Katti, S.K., D.M. LeMaster and H. Eklund. 1990. Crystal structure of thioredoxin from *E. coli* at 1.68 Angstroms resolution. **J. Mol.Biol**. 212: 167-184.

Kaden, V., E. Lange., U. Fischer and G. Strebelow. 2000. Oral immunisation of wild boar against classical swine fever: evaluation of the first field study in Germany. **Vet Microbiol**. 73: 239-252.

Kern, B., K.R. depner., W. Letz., M. Rott., S. thalheim., B. Nitschke., R. Plagemann and B. Liess. 1999. Incidence of classical swine fever (CSF) in wild boar in a densely populated area indicating CSF virus persistence as a mechanism for virus perpetuation**. Zentralbl Veterinarmed B**. 46: 63-67.

Kesik, M., V. Saczynska., B. Szenczyk and A. Pluciennicza k. 2004. Inclusion bodies from recombinant bacteria as a novel system for delivery of vaccine antigen by the oral route. **Immunology Letters**. 91: 197-204.

Klinkenberg, D., R.J. Moormann., a.J. de Smit., A. Bouma and M.C. de jong. 2002. Influence of maternal antibodies on efficacy of a subunit vaccine: transmission of classical swine fever virus between pigs vaccinated at 2 weeks of age. **Vaccine.** 20: 3005-3013.

Knoetig, S.M., A. Summerfield., M. Spagnuolo-Weaver and K.C. McCullough. 1999. Immunopathogenesis of classical swine fever: role of monocytic cells. **Immunology**. 97: 359-366.

Koenen, F., G. Van Caenegem., J.P. Vermeersch., J. Vandenheede and H. Deluyker. 1996. Epidemiological characteristics of an outbreak of classical swine fever in an area of high pig density. **Vet Rec**. 139: 367-371.

Konig, M., T. Lengsfeld., T. Pauly., R. Stark and H.J. thiel. 1995. Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins. **J Virol.** 69: 6479-6486.

Kool, M., J.W. Voncken., F.L. van Lier., J. Tramper and J.M. Vlak. 1991. Detection and analysis of Autographa californica nuclearpolyhedrosis virus mutants with defective interfering properties. **Virology.** 183: 739–746.

Krell, P. J. 1996. Passage effect of virus infection in insect cells. **Cytotechnology** 20: 125–137.

Kummerer, B.M., N. tautz., P. Becher., H. Thiel and G. Meyer. 2000. The genetic basis for cytopathogenicity of pestiviruses. **Vet Microbiol**. 77: 117-128.

Laddomada, A., 2000. Incidence and control of CSF in wild boar in Europe. **Vet Microbiol**. 73: 121-130.

Lai, V.C.H., C. Cheng Kao., E. Ferrari., J. Park., A. S. Uss., J. Wright-Minogue., Z. Hong and J.Y. N. Lau. 1999. Mutational Analysis of Bovine Viral Diarrhea Virus RNA-Dependent RNA Polymerase **J. Virol**. 73: 10129-10136.

Langedijk, J.P., W.G. Middel., R.H. Meloen., J.A. Kramps and J.A. de Smit. 2001. Enzyme-linked immunosorbent assay using a virus type-specific peptide based on a subdomain of envelope protein Erns for serologic diagnosis of pestivirus infections in swine. **J Clin Microbiol.** 39: 906-912.

Le, S.Y., N. Sonenberg and J. V. Maizel. 1995. Unusual folding regions and ribosome landing pad within hepatitis C virus and pestivirus RNAs. **Gene.** 154: 137-143.

Lee, H. Y. and P.J. Krell. 1992. Generation and analysis of defective genomes of Autographa californica nuclear polyhedrosis virus. **J Virol**. 66: 4339–4347.

Lee, N. 1980. Molecular aspects of *ara* regulation, pp. 389-410. In J.H.Miller and W.S. Reznikoff, eds. **The operon**. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Lee, N., C. Francklyn and E.P. Hamilton. 1987. Arabinose-Induced binding of AraC protein to *ara*I2 Activates the *ara*BAD Operon Promoter. **Proc.Natl.Acad.Sci.USA**. 84: 8814-8818.

Liljeqvist, S., D. Haddad., K. Berzins., M. Uhlen and S. Stahl.1996. A Novel Expression System for *Salmonella typhimurium* Allowing High Production Levels, Product Secretion and Efficient Recovery. **Biochem. Biophy. Res. Commun**.218: 356-359.

Lohmann, V., F. Korner., U. Herian and R. Bartenschlager. 1997. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. **J. Virol**. 71: 8416-8428.

Lowings, P., G. Ibata., G.M. De Mia., D. Rutili and D. Paton. 1999. Classical swine fever in Sardinia: epidemiology of recent outbreaks**. Epidemiol Infect**. 122: 553-559.

Lu, Z., E.A. Diblasio-Smith., K.L. Grant., N.W. Warne., E.R. La Vallie., L.A. Collins-Racie., M. T. Follettie., M.J. Williamson and J.M. Mccoy. 1996.

Histidine patch thioredoxins**. J. Biol. Chem**. 271: 5059-5065.

Martin-Gallardo, A., E. Fleischer., S.A. Doyle., R. Arumugham., P.L. Collins., S.W. Hildreth and P.R. Paradiso. 1993. Expression of the G glycoprotein gene of human respiratory syncytial virus in Salmonella typhimurium. **J. Gen. Virol**. 74: 453 - 458.

Markowska-Daniel, I., R.A. Collins and Z. Pejsak. 2001. Evaluation of genetic vaccine against classical swine fever. **Vaccine.** 19: 2480-2484.

Mason, H and S. Arntzen., 1995. Transgenic plants as vaccine production systems. **Trends Biotechnol**. 13:388–392.

Mason, H.S., D.M. Lam and C.J. Arntzen, 1992. Expression of hepatitis B surface antigen in transgenic plants. **Proc. Natl. Acad. Sci. USA**. 89: 11745–11749.

Mason, H.S., J.M. Ball., J.J. Shi., X. Jiang., M.K. Estes and C.J. Arntzen. 1996. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. **Proc. Natl. Acad. Sci. USA**. 93: 5335–5340.

McGoldrick, A., E. Bensaude., G. Ibata., G. Sharp and D.J. Paton. 1999. Closed one-tube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. **J Virol Methods**. 79: 85-95

Meyer, D., M.A. Hofmann and J.D.Tratschin. 2004. Attenuation of classical swine ever virus by deletion of the viral Npro gene. **Vaccine**. 22: 317-328.

Meyer, G., T. Rumenapf and H.J. Thiel. 1989. Molecular cloning and nucleotide sequence of the genome of hog cholera virus. **Virology**. 171: 555-567.

Meyer, G., H.J. Thiel and T. Rumenapf. 1996. Classical swine fever virus: recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. **J Virol**. 70: 1588-1595.

Meyer, G and H.J. Thiel. 1995. Cytopathogenicity of classical swine fever virus caused by defective interfering particles. **J. Virol**. 69: 3683-3689.

Mikiewicz, D., B. Wrobel., G. wecgrzyn and A. Piucienniczak. 1997. Isolation and Characterization of a ColE1-like Plasmid from *Enterobacter agglomerans* with a Novel Variant of *rom* Gene. **Plasmid**. 38: 210-219.

Moennig, V. 2000. Introduction to classical swine fever: virus, disease and control policy. **Vet Microbiol**. 73: 93-102.

Moennig, V., G. Floegel-Niesmann and I. Greiser-Wilke. 2003. Clinical signs and epidemiology of classical swine fever: a review of new knowledge. **Vet J**. 165: 11-20.

Moennig, V and P.G. Plagemann. 1992. The pestiviruses. **Adv Virus Res**. 41: 53-98.

Moormann, R.J., A. Bouma., J.A. Kramps., C. Terpstra and H.J. Smit. 2000. Development of a classical swine fever subunit marker vaccine and companion diagnostic test. **Vet. Microbiol**. 73: 209-219.

Moormann, R.J., H.G. Van gennip., G.K. Miedema., M.M. Hulst and P.A. van Rijn. 1996. Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. **J Virol**. 70: 763-770.

Moser, C., N. Ruggli., J.D. Tratschin and M.A. Hofmann. 1996. Deletion of antibodies against classical swine fever virus in swine sera by indirect ELISA using recombinant envelope glycoprotein E2. **Vet. Microbiol**. 51:41-53.

Muyldermans, G., M.C. San Gabriel., A. Caij., A. De Smet and R. Hamers. 1993. Polymerase chain reaction-mediated cloning and in vitro translation of the genes coding for the structural proteins of hog cholera virus. **Arch Virol.** 132: 429-435.

Paton, D.J and I. Greaser-Wike. 2003. Classical swine fever--an update. **Res Vet Sci.** 75: 169-178.

Paton, D.J., A. Mcglodrick., E. Bensaude., S. Belak., C. Mittelholzer., F. Koenen., H.

Vanderhallen., I. Greiser-Wike., H. Scheibner., T. Stadejek., M. Hofmann and B. Thuer. 2000. Classical swine fever virus: a second ring test to evaluate RT-PCR detection methods. **Vet Microbiol**. 77: 71-81.

Peeters, B., K. Bienkowska-Szewczyk., M. Hulst., a. Gielkens and T. Kimman. 1997. Biologically safe, non-transmissible pseudorabies virus vector vaccine protects pigs against both Aujeszky's disease and classical swine fever. **J Gen Virol**. 78: 3311-3315.

Pierce. 2005. **Modified Lowry protein assay kit: Instructions**. Pierce Biotechnology Inc. USA.

Pijlman, G. P., E. van den Born., D.E. Martens and J.M. Vlak. 2001. Autographa californica baculoviruses with large genomic deletions are rapidly generated in infected insect cells. **Virology.** 283: 132–138.

Pissarra, P. de N. 2000. Recombinant DNA Proteins for the Biopharmaceutical Industry and the Future for *Escherichia coli*. **Nature Biotechnology**. 18: 831-833.

Possee, R. D. 1997. Baculoviruses as expression vectors. **Curr Opin Biotechnol**. 8: 569–572.

Qiagen. 2001. **The QIA expressionist**: A hand book for high-level expression and

purification of 6X His-taqqed proteins. Qiagen Inc.USA.

Ressang, A.A. 1973. Studies on the pathogenesis of hog cholera. I. demonstration of hog cholera virus subsequent to oral exposure. **Zentralbl. Veterinaermed. Beih**. 20: 265-271.

Ruggli, N., J.D. Tratschin., C. Mittelholzer and M.A. Hofmann. 1996. Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. **J Virol.** 70: 3478-3487.

Sambrook, J and D.W. Russell. 2001. **Molecular Cloning: a laboratory Manual**. 3 nd ed. Cold Spring Harbour Laboratory Press. New York.

Schneider, R., G. Under., R. Stark., E. Schneider-Schezer and H.J. Thiel. 1993. Identification of a structural glycoprotein of an RNA virus as a ribonuclease. **Science.** 261: 1169-1171.

de Smit, A.J. 2000. Laboratory diagnosis, epizootiology, and efficacy of marker vaccines in classical swine fever: a review. **Vet Q**. 22: 182-188.

de Smit, A.J., A. Bouma., C. Terpstra and J.T. van Oirschot. 1999. Transmission of classical swine fever virus by artificial insemination. **Vet Microbiol**. 67: 239-249.

de Smit, A.J., A. Bouma., E.P. de Kluijver., C. Terpstra and R.J. Moormann. 2000. Prevention of transplacental transmission of moderate-virulent classical swine fever virus after single or double vaccination with an E2 subunit vaccine. **Vet Q**. 22:150.

de Smit, A.J., P.L. Eble., E.P. de Kluijver., M. Bloemraad and A. Bouma. 1999. Laboratory decision-making during the classical swine fever epidemic of 1997-1998 in the Netherlands. **Prev Vet Med**. 42: 185-199.

Sambrook, J., E.F. Fritsch and T. maniatis. 1989**. Molecular Cloning: a Laboratory Manual**. 2nd ed., Cold Spring Harbor Laboratory Press.

Sorenson, H. P. and Mortensen, K. K. 2005. Advanced genetic strategies for recombinant protein expression in *Escherichia coli.* **J. of Biotech**., 115: 113-128.

Steffens, S., H.J. Thiel and S.E. Behrens. 1999. The RNA-dependent RNA polymerases of different members of the family *Flaviviridae* exhibit similar properties *in vitro.* **J. Gen. Virol**., 80: 2583 – 2590

Stegeman, A., A.R. Elbers., J. Smak and M.C. de Jong. 1999. Quantification of the transmission of classical swine fever virus between herds during the 1997-1998 epidemic in The Netherlands. **Prev Vet Med**. 42: 219-234.

Stegeman, A., A.R. Elbers., A. Bouma., H. de Smit and M.C. de Jong. 1999. Transmission of classical swine fever virus within herds during the 1997-1998 epidemic in The Netherlands. **Prev Vet Med**. 42: 201-218.

Stewart, W.C. 1981. **Hog cholera. In disease of swine**. 5th ed. A.D. Leman, R.D.Glock, W. L. Mengeling, R.H.C.Penny, E.Scholl,and B.Straw. Ames: Iowa State Univ Press.

Sudbery, P.E., 1996. The expression of recombinant proteins in yeasts. **Curr. Opin. Biotechnol.** 7: 517–524.

Summerfield, A., M.A. Hofmann and K.C. McCullough. 1998. Low density blood granulocytic cells induced during classical swine fever are targets for virus infection. **Vet Immunol Immunopathol**. 63: 289-301.

Summerfield, A., K. Zingle., S. Inumaru and K.C. McCullough. 2001. Induction of apoptosis in bone marrow neutrophill-lineage cells by classical swine fever virus**. J. Gen. Virol**. 82: 1309-1318.

Susa, M., M. Konig., A. Saalmuller., M.J. Reddehase and H.J. Thiel. 1992. Pathogenesis of classical swine fever: B-lymphocyte deficiency caused by hog cholera virus. **J Virol.** 66: 1171-1175.

Tacket, C.O., H.S. Mason., G. Losonsky., J.D. Clements., M.M. Levine and C.J.

Arntzen. 1998. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. **Nat. Med.** 4: 607–609.

Tautz, N., K. Elbers., D. Stoll., G. Meyers and H.J. Thiel. 1997. serine protease of pestiviruss: determination of cleavage sites. **J. Virol**. 71: 5415-5422.

Thanavala, Y., Y.F. Yang., P. Lyons., H.S Mason and C. Arntzen. 1995. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. **Proc. Natl. Acad. Sci. USA.** 92: 3358–3361.

Thiel, H.J., R. Stark., E. weiland., T. Rumenapf and G. Meyers. 1991. Hog cholera virus: molecular composition of virions from a pestivirus. **J Virol**. 65; 4705-4712.

Uttenthal, A., T. Storgaard., M.B. Oleksiewicz and K. de Stricker. 2003. Experimental infection with the Paderborn isolate of classical swine fever virus in 10-week-old pigs: determination of viral replication kinetics by quantitative RT-PCR, virus isolation and antigen ELISA. **Vet Microbiol.** 92: 197-212.

Vlasova, A., T. Grebennikova., A. Zaberezhny., I. Greiser-Wilke., G. Floegel-Niesmann., V. Kurinnov.,T. Aliper and E. Nepoklonov. 2003. Molecular Epidemiology of Classical Swine Fever in the Russian Federation**. J. Vet. Med.** 50: 363–367.

Van Oirschot, J.T.1980. **Persistent and inapparent infections with swine fever**

virus of low virulence : Their effects on the immune system. Thesis, stata UniV Utrecht.

Van Oirschot, J.T and C. Terpstra. 1977. A congenital persistent swine fever infection. I. Clinical and virological observations. **Vet Microbiol**. 2: 121-132.

Van Oirschot, J.T. 1999. **hog cholera. In disease of swine**. 8th ed. Iowa state University Press.

Van Rijn, P.A., A. Bosser., G. Wenvoort and R.J. Moormann. 1996. Classical swine fever virus (CSFV) envelope glycoprotein E2 containing one structural antigenic unit protects pigs from lethal CSFV challenge. **J Gen Virol**. 77: 2737-2745.

Van Rijn, P.A., G.K. Miedema., G. Wensvoort and H.G. van Gennip. 1994. Antigenic structure of envelope glycoprotein E1 of hog cholera virus. **J Virol**. 68: 3934-3942.

Van Rijn, P.A., H.G. van Gennip and R.J. Moormann. 1999. An experimental marker vaccine and accompanying serological diagnostic test both based on envelope glycoprotein E2 of classical swine fever virus (CSFV).**Vaccine.** 17: 433-440.

Van Zijl, M., G. Wensvoort., E. de Kluyver., M. Hulst., H. van der Gulden., A.

Gielkens., A. berns and R. moormann. 1991. Live attenuated pseudorabies virus expressing envelope glycoprotein E1 of hog cholera virus protects swine against both pseudorabies and hog cholera. **J Virol.** 65: 2761-2765.

Vialard, J. E., B.M. Arif and C.D. Richardson. 1995. Introduction to the molecular biology of baculoviruses. **Methods Mol Biol**. 39: 1–24.

Wallace, B.J and S.R. Kushner. 1984. Genetic and physical analysis of the thioredoxin (trxA) gene of *E. coli* K-12. **Gene**. 32: 399-408.

Warrener, P and M.S. Collett. 1995. Pestivirus NS3 (p80) protein possesses RNA helicase activity**. J. Virol**. 72: 7681-7684.

Weickert, M.J., D.H. Doherty., E.A. Best and P.O. Olins. 1996. Optimization of heterologous protein production in *Escherichia coli*. **Curr Opin Biotechnol**. 7: 494-499.

.

Wensvoort, G., J. Boonstra and B.G. Bodzinga. 1990. Immuno-affinity purification and characterization of the envelope protein E1 of hog cholera virus. **J. Gen Virol.** 71: 531-540.

Wickham, T. J., T. Davis., R.R. Granados., D.A. Hammer., M.L. Shuler and Wood, H. A. 1991. Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. **Biotechnol Lett** 13: 483–488.

William, D.R and D. Matthews. 1988. Outbreaks of classical swine fever in Great Britain in 1986. **Vet Rec.** 122: 479-483.

Yu, X., C. Tu., H. Li., R. Hu., C. Chen., Z. Li., M. Zhang and Z. Yin. 2001. DNA-mediated protection against classical swine fever virus. **Vaccine.** 19: 1520-1525.

APPENDIX

APPENDIX A

Chemical Reagents and Substances

1. Bacterial Media and Solution

- Luria Bertani (LB) medium (broth)

  10 g/litre tryptone

  5 g/litre yeast extract

  10 g/litre NaCI

- LB agar

  LB medium containing 15 g/litre agar

- Kanamycin stock solution

  25 mg/ml in H_20. sterile filter, store in aliquots at -20 °C

- Arabinose stock solution

  20% in water store in aliquots at -20 °C

2. Buffer for Agarose Gel Electrophoresis

- 20X TAE buffer pH 8.3 (1 litre)

  0.8 M Tris ; 96.9 g

  0.4 M Sodium acetate; 32.8 g of $NaOAc\text{-}3H_20$

  0.04 M Na_2EDTA; 14.9 g

  Adjust pH with glacial acetic acid to pH 8.3 and bring to 1 litre

with distilled water.

- 10X loading buffer / dye

  20% glycerol

  0.01% Bromphenol blue

  add TE to final volume

- 5 mg/ml ethidium bromide (EtBr)

  500 mg EtBr

  add distilled water to 100 ml

3. <u>Buffers for Protein Purification</u>

Lysis buffer B (1 litre):

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 litre):

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 litre):

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 litre):

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 : Due to the dissociation of urea, the pH of buffer should be adjusted immediately prior to use. Do not autoclave.

4. <u>SDS-PAGE Reagents</u>

- 2x SDS-PAGE sample buffer

2.5 ml of 4xTris Cl 1 SDS, pH 6.8 (250 mM Tris Cl)

2.0 mI 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 Cl/SDS pH 6.8, buffer for stacking gel (250 ml)

0.5M TrisCl ; 15 g TrisCl

0.4% SDS; 1 g SDS

Distilled water 200 ml)

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

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

1.5M TrisCl; 91 g Tris Base

0.4% SDS; 2 g SDS

Distilled water 400 ml

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

- 30% Acrylamide : Bis (100 ml)

40% (19:1) acrylamide:Bis solution (Accugel ^{TM}) 75 ml

add distilled water to 100 ml

- 10% Ammonium persulfate

100 mg Ammonium persulfate

1 ml distilled water

- TEMED (N,N,N',N'-tetramethylethylenediamine)

store protected from light at 4°C

- Glycine buffer

192 mM glycine

25 mM Tris base

0.1% SIDS

- Coomasie Brilliant Blue strain (100 ml)

0.25 g Coomasie brilliant blue powder

45 ml methanol

10 ml acetic acid

45 ml distilled water

- Destrainning solution (100 ml)

45 ml methanol

10 ml acetic acid

45 ml distilled water

5. lmmunoblotting Reagents

-10x PBS

80 g NaCl

2 g KCl

11.5 g Na_2HPO_4

2 g KH_2PO_4

add distilled water to 1 litre

- Blocking agent

5% skim milk in PBS

- DAB substrate (1 ml)

6 mg DAB

10 µl H_2O_2

990 µl PBS

- Transfer buffer (1 litre)

25mM Tris; 3 g Tris base

190mM glycine; 14.4 g glycine

20% Methanol ; 200 ml Methanol

Distilled water adjust to 1 liter

APPENDIX B

The standard methods

1. Single Step Competent cells preparation for Transformation (Bio Basic Inc.)

1. add 1 ml of cells at A_{600nm} = 0.5-0.7 in 1.5 ml microtubes.
2. Spin at 4000 rpm for 4-5 min and then remove the supernatant.
3. Add 100 µl of pre-cold SSCS and gently mix the cells. Aliquot cell mixture. It is ready to use or can be stored at -70 ^{o}C for future use.

4. Add 100 pg to 10 ng of transforming DNA to the cells.
5. Mix the cells and DNA and place on ice for 10 min, then 37 ^{o}C for 5 min, then on ice for 10 min.

6. Add 1 ml of LB and incubate the tube at 37 ^{o}C for 1 hour.
7. Plate the cells on the appropriate selective or differential media.

2. Transformation (Ausubel *et al.*, 1995)

1. Thaw the competent cell on ice, for 30 minutes.
2. Mix the constructed plasmid from ligation to the competent cell, stand on ice for 30- 60 minutes.

3. Heat shock the cell at 42°C in the heat block, and immediately place tube on ice.

4. Add the SOC medium 1 ml and incubate with shaking at 37°C for 1 hour.

5. Centrifuge the culture at 6,000 rpm for 5 minutes.

6. Spread the cells on the prewarmed LB plate containing 100 ug/ml ampicillin , air dry plate, and incubate overnight at 37°C

7. The recovery clone of *E. coli* with recombinant plasmid was determined by PCR assay, restriction endonuclease, and DNA sequencing.

3. Ligation (Sambrook *et at.*, 1989)

The following reaction conditions are for ligation of DNA inserts with cohesive ends to DNA vectors with complementary cohesive ends to produce circular recombinant molecules. A molar ratio of 3:1 insert:vector was used.

1. Add the following component to the 0.5 ml microtube
   - 5x ligase buffer 4 ul
   - vector DNA : insert DNA (1:3)
   - autoclaved distilled water to 20 ul

2. Add 1.0 µl (0.1 unit) of T4 DNA ligase (Promega®, Inc.). Mix gently and then breifly spin down to bring the content to the bottom of the tube.

3. Incubate at 16 °C overnight.

4. The ligation reaction was used in transformation to competent cell of *E. coli* 5 -10 µl each reaction.

4. <u>Rapid Alkaline Extraction Method for Plasmid Isolation</u> (Birnboim, 1983)

1. From overnight cultures of *E. coli* in LB broth, pour 1.5 ml into 1.5 ml microtube.
2. Centrifuge for 5 minutes, at 5,000 rpm and carefully aspirate off the medium.
3. Add 100 µl of solutionI and resuspend by vortexing until the suspension become homogenous.

4. Add 200 µl of solutionII and gently inverse the tube up side down. The cells should lyse and turn somewhat clear and viscous.

5. Let it stand for 3 minutes (should not more than 5 minutes) and then add 150 µl of solution III. Mix again by inversion until a white clot of DNA/protein/SDS form. Incubate on ice 10 - 30 minutes. For the better precipitation of the clot. add 20µ l of chloroform to the suspension, mix well.

6. Centrifuge at 13,000 rpm for 5 minutes.
7. Pour off the supernatant (~400 µl) into a fresh 1.5 ml microtube. Further cleanup can be accomplished with phenol / chloroform extractions.

8. For DNA precipitation, add two volume of absoluted ethanol ~1 ml and gently inverse tube up side down. Keep it in -80°C for 30 minutes.

9. Centrifuge at 13,000 rpm for 10 - 15 minutes. Carefully pour off ethanol.
10. Wash the pellet with 0.5 - 1 ml of 75% ethanol. Air dry the pellet.
11. Resuspend pellet in 20 - 50 µl of TE (volume dependent on copy number of plasmid).

5. <u>Restriction Endonuclease Assay</u> (Fermentus®)

This procedure is used for cutting the target DNA with two enzymes (Double digestion method).

1. Isolate the plasmid from *E. coli* or prepared the target DNA.
2. Add the following components to 1.5 ml microtube;
   - target DNA 10 µl (in TE & distilled water), the volume depends on the concentration of DNA.
   - Y+Tango® 1X buffer for 2 µl
   - 2-fold of Hind III and 1-fold of XbaI restriction enzyme add distilled water to desirable volume (30 µl)
3. incubate at 37 °C for 3 hours to complete enzyme reaction.
4. Purify the target DNA by agarose gel electrophoresis and DNA extraction from agarose gel.

6. Phenol-Chloroform Extraction of RNA and Ethanol Precipitation (Sambrook and Russell, 2001)

1. RNA was extracted from 100 µl of allantoic fluids that it was 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 0.5µ l 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. Wash the RNA pellet with 75% ethanol. Centrifuge at 13.000 rpm for 5 minutes. Decant the supernatant, and dry the pellet by air.

7. Agarose Gel Electrophoresis

1. Prepare an agarose gel, according to recipes listed below, by combining the agarose

(low gel temperature agarose may also be used) and water in a 250 ml) Ehrlenmeyer flask, and heating in a microwave for 2 - 4 minutes until the agarose is dissolved.

2. Pour the gel onto a taped plate with casting combs in place. Allow 20 - 30 minutes for solidification.

3. Carefully remove the tape and the gel casting combs and place the get in a horizontal electrophoresis apparatus. Add 1x TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose get.

4. Add at least one- tenth volume of 10x agarose gel loading dye to each DNA sample, mix, and load into the wells. Electrophoresis the gel at 50 - 100 V/cm until the required separation has been achieved. Visualize the DNA fragments on a long wave UV light box.

8. <u>SDS-Polyacrylamide Get Electrophoresis (SDS-PAGE)</u> (Cochet *et al.*, 1998)

8.1 Prepare the gel by the recipe as follow for 2 gels

- Seperating gel (12%)

H_20	2.8 ml
4x TrisHCI/SDS pH 8.8.	2.0 ml
30% Acry/0.8%bis-Acryl	3.2 ml
10% APS	26.6 ul

TEMED	5.3 ul

- Stacking get

H_20	3.05 ml
4x TrisHCI/SDS pH 6.8	1.25 ml
30% Acryl/0.8%bis-Acryl	0.65 ml
10% APS	25 ul
TEMED	5 ul

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

8.3. Apply 5 - 25µ l (1 - 10ug total protein) of cell lysate to each well of a 0.75-1.0 mm thick gel.

8.4. Run electrophoresis (100 volt, 70 minutes, constant ampere)

9. <u>Western Transfer and Blotting</u> (Cochet *et al.*, 1998)

1. Cut the nitrocellulose membrane with sharp blade to the expected size
2. Soak the gel (from SDS-PAGE), the nitrocellulose membrane, the whatmann papers, and sponges in cool transfer buffer for 10 minutes.

3. Set the transfer apparatus, then transfer proteins from gel to nitrocellulose membrane at 400 mAmp for 6 hours in transfer buffer (25mM Tris base, 190mM

glycine, 20% MeOH). The transfer set should be placed in ice box to control the temperature.

4. Remove the blot from the transfer apparatus immediately place into blocking buffer (5% skim milk in 1x PBS+ 0.5 % Tween 20) and incubate for 1 hour at room temperature with gently agitation (optional 2 hour at 37°C or overnight at 4°C).

5. Dilute the primary antibody (goat-anti H5N1 avian influenza virus hyperimmune sera absorb with supernatant of virus wild type (1:50) or mouse IgG anti-histidine monoclonal antibody (1:3,000). Decant the blocking buffer from the blot, add the primary antibody solution, and incubate with agitation for one hour at room temperature (60 minutes at 37°C)

6. Wash 3 times with agitation in PBS-0.5% Tween for 5 - 10 minutes each.
7. Dilute the secondary antibody (rabbit anti-goat IgG (1:1,000) or goat anti-mouse IgG (1:500) conjugated with peroxidase). Decant the primary antibody from the blot, add the secondary antibody solution, and incubate with agitation for one hour at room temperature (45 minutes at 37°C)

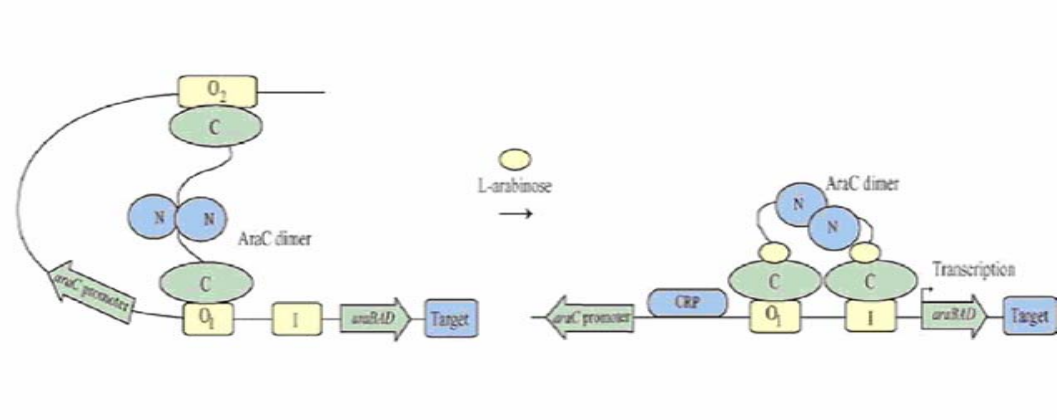
8. Decant the secondary antibody solution. Wash 3 times with agitation in PBS 0.5 % Tween for 5 - 10 minutes each.

9. Add DAB substrate and incubate for 5 - 15 minutes.
10. To stop enzyme-substrate reaction, place the blot in distilled water.

10. Nucleotide Base Abbreviations Used in Nucleic Acid Sequences

A	adenosine
C	cytidine
G	guanine
T	thymidine

APPENDIX C



Appendix Figure C1 Regulation of the arabinose operon in *E. coli*

CURRICULUM VITAE

NAME : **Miss. Damratsamon Surangkul**

BIRTH DATE : **March 18, 1972**

BIRTH PLACE : **Phitsanuloke, Thailand**

EDUCATION : **YEAR INSTITUTION DEGREE/DIPLOMA**

: **1994 Chiangmai Univ. Bachelor's Degree**

: **1998 Chiangmai Univ. Master's Degree**

POSITION/TITLE : **Lecturer**

WORK PLACE : **Naresuan University**

SCHOLARSHIP/AWARDS: -