CLONING AND EXPRESSION OF ORF7 GENE OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

INTRODUCTION

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is a virus currrenty classified as a member of the order of *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Meulenberg, 2000). PRRSV is characterized by reproductive failure of sows and respiratory problems of piglets and growing pigs. The reproductive failure is characterized by infertility, late fetal mummification, abortions, stillbirths, and the birth of weak piglets that often die soon after birth from respiratory disease and secondary infections (Keffaber, 1989, Bilodeau *et al*., 1991, Christianson *et al*., 1992, OIE, 2004). The PRRSVs are divided into two strains: European strain, and North American strain, on the basis of antigenic differences in nucleocapsid proteins (encoded by ORF7 gene) and matrix (M) proteins (encoded by ORF6 gene) (Dea *et al*,.2000).

The virus can be isolated from tissues such as serum, ascitic fluids, or organ samples, such as lungs, tonsil, lymph nodes and spleen of affected pigs. The porcine alveolar macrophages are the most susceptible culture for virus. MARC-145 cells are also suitable (OIE, 2004).

Diagnosis of the PRRSV infection is difficult to achieve in tissues because they rarely have microscopic lesions or non-diagnostic (Benfield *et al*., 1999). The virus is identified and characterized by immunostaining with specific antisera. Reverse transcription Polymerase Chain Reaction, immunohistochemistry, and *in situ* hybridization have been confirmed the PRRSV infection. Several serological tests, enzyme linked immunosorbent assays (ELISAs) (the most suitable technique) such as indirect ELISA or blocking ELISA or a double ELISA, immunoperoxidase monolayer assay (IPMA), indirect fluorescent antibody test (IFA), and serum neutralizing test (SNT) are available for the detection of serum antibodies to PRRSV (Taylor, 1995).

Vaccination against PRRS can result in protective immunity (Hesse *et al*., 1996, Benfield *et al*., 1999) but it is only recommended for pigs in PRRSV-positive herds (OIE, 2004). The pregnant sows and gilts and the breeding age boars are not vaccinated with modified live virus vaccines. Modified live virus vaccines can spread to nonvaccinated pigs (Torrison *et al*., 1996, Botner *et al*., 1997) and cause vaccine-virus-induced PRRS reproductive failure in the affected herds (Botner *et al*.,1997, Madsen *et al*., 1998).

In this thesis, ORF7 genes of European and North American strains have been cloned. Their recombinant nucleoproteins were produced in *E.coli* by the pBAD directional TOPO^{TM} expression system. These recombinant nucleoproteins may be suitable for diagnosis of PRRSv in Thailand. The method for cloning and expression of ORF7 gene of PRRSVs will be described.

OBJECTIVES

1. To develop Polymerase Chain Reaction technique for the differentiation of the strain of Porcine Reproductive and Respiratory Syndrome virus (PRRSV)

2. To clone and express ORF 7 of the PRRSV and to determine the potential usage of the recombinant protein for the detection of PRRSV

LITERATURE REVIEWS

1. Porcine Reproductive and Respiratory Syndrome (PRRS)

1.1 Background of Porcine Reproductive and Respiratory Syndrome

Porcine Reproductive and Respiratory Syndrome (PRRS) is characterized by reproductive failure of sows and respiratory problems of piglets and growing pigs. PRRS was first reported in the United States in 1987 (Keffaber, 1989; Benfield *et al.*, 1999). PRRS were recognized in Canada in autumn of 1987 (Dea *et al.*, 1990; Bilodeau *et al.*, 1991; Dea *et al*., 1992), in Japan in 1989 (Kuwahara *et al*., 1994; Murakami *et al*., 1994; Shimizu *et al*, 1994), in Germany in 1990 (Lindhaus and Lindhaus, 1991), in the Netherlands, Spain, France and the United Kingdom in 1991 (Wensvoort *et al*.,1991; White, 1991; Meredith,1992), in Denmark in 1992 (Botner *et al*., 1994), and since 1992 in much of the rest of the world wherever large numbers of swine are raised (Meredith, 1995). There are many names of the PRRS such as mystery swine disease (often used in the United States before the cause of PRRS was identified), blue ear disease (based on transient, bluish discoloration of the ears of some affected sows and gilts), swine infertility and abortion syndrome, and porcine epidemic abortion and respiratory syndrome (Taylor, 1995). PRRS occurs in most major pig-producing areas throughout the world. The reproductive failure is characterized by infertility, late fetal mummification, abortions, stillbirths, and the birth of weak piglets that often die soon after birth from respiratory disease and secondary infections. Older pigs may demonstrate mild signs of respiratory disease, sometimes complicated by secondary infections. Animals other than pigs do not seem to be affected by PRRS (OIE, 2004).

Although epidemiological data from even the very early cases of PRRS suggested that it was an infectious disease, it was not until several years later that the cause, PRRS virus (PRRSV), was identified with certainty (Wensvoort *et al*., 1991; Collins *et al*., 1992). The origin of PRRSV is still unknown, but it is intriguing that North American and European isolates of the virus have marked genotypic and

phenotypic differences, suggesting that if they originated from a common ancestor, they developed along different evolutionary lines.

PRRS continues to plague the swine industry both as an endemic respiratory disease, often as part of what has been referred to as the porcine respiratory disease complex, and as a sporadic, acute reproductive disease most strikingly manifested as an unusually high incidence of abortions. Recently, severe epidemics of reproductive disease described in the United States as acute or atypical PRRS (Halbur and Bush, 1997; Epperson and Holler, 1997; Mengeling *et al*., 1997; Zimmerman *et al*., 1997) and caused by what may be more virulent strains of PRRSV, suggest that PRRSV is continuously changing a concept that is supported in part by base sequence (Kapur *et al*., 1996; Murtaugh *et al*., 1997) and restriction endonuclease analysis (Wesley *et al*., 1996; Mengeling *et al*., 1997). If so, the potential for genotypic and related phenotypic change, and the ability of PRRSV to persist in herds for extended periods of time, serious challenges for the control and possible eradication of PRRS.

1.2 Clinical signs

Clinical signs of PRRS are extremely variable and influenced by strain of virus (Halbur *et al*., 1996), immune status of the herd (Keffaber. 1989; Wensvoort, 1993), and management factors (Blaha, 1992; White, 1992). Clinical disease in a herd is primarily the consequence of acute viremia in individuals (Pol *et al*., 1991; Terpstra *et al*., 1991; Collins *et al*., 1992) and transplacental transmission of virus from viremic dams to their fetuses (Terpstra *et al*., 1991), which occurs most efficiently in the third trimester of pregnancy (Christianson *et al*., 1993; Mengeling *et al*., 1994)

Strains of PRRSV vary remarkably in virulence (Halbur *et al*., 1996). Low-virulence strains can cause completely subclinical epidemic or endemic infections of herds (Morrison *et al*., 1992), whereas highly virulent strains can cause severe clinical illness. In sows, there is usually an inappetance lasting 24-36 hours – 4 days in individuals and 7-10 days in the herd. Sows may be listless with labored breathing but fever is not consistent, rarely exceeds 40^{o}c and is transient, often lasting a single day.

Abortion may occur at any stage of gestation, and is first apparent at 22 days post infection (p.i.), but most cases occur later. There may be accompanying skin changes such as hyperemia or congestion of the ears, nose and tail, but only 1-2% of animals develop these changes. Farrowing problems develop with a rise in stillbirths and increased mortality in piglets aged up to 1 week. This is partially due to premature farrowing. Lactation is affected in some sows and inappetance on lactation may lead to anoestrus. Returns to service may also occur although this may be partly attributed to boar fertility. Boars may be listless and occasional animals develop blue ears. A drop in semen quality may occur but cannot be linked in every case to clinical signs.

In piglets, antenatal infection or effects on the sow may lead to an increase in stillbirths and antepartum deaths appearing as large mummified piglets. Many of the piglets born are weak, may have splayleg and do not survive. Affected piglets may give edema of the eyelids which gives a spectacled appearance and there may be conjunctivitis. Hemorrhage into the gut may lead to melaena and the appearance of a grayish diarrhea. Labored breathing may also be apparent (Gordon, 1992; Hopper *et al*., 1992; White, 1992). In weaners and growers, there is an increase in respiratory disease and skin changes may be seen in recovering pigs, 5-7 days after infection (Solano *et al*., 1995; Van Alstine *et al*., 1996).

In the herd, the first 8 weeks of the disease are characterized by a rise in pre-weaning mortality and a dramatic rise in the number of stillbirths. Later, mummified piglets appear and live births decrease. Post-weaning mortality may increase and the number of treatments required increases markedly. In the second period of 6 weeks, there is an overeall improvement but service increase. Illness in piglets and weaned pigs remains common and numbers of treatments are still raised. In the third period, a drop in total births occurs as a result of continuing low numbers born and the increase in returns to service. A return to near normal levels of production can be expected 26 weeks after infection (Albina *et al*., 1994; Dee and Joo, 1994; Stevenson *et al*., 1994; Benfield *et al*., 1997).

1.3 Pathogenesis

The pathogenesis of PRRSV infection is based on PRRSV infection and replication within cells of the monocyte/macrophage lineage (Wensvoort *et al*., 1991; Voicu *et al*., 1994; Rossow *et al*., 1995; Rossow *et al*., 1996; Molitor *et al*., 1996). Exposure of a mucosal surface to PRRSV results in viremia within 12 hours after infection (Rossow *et al*., 1995), and PRRSV antigen is identified in macrophages of the nasal mucosa, lung, and tonsil (Rossow, 1996). Sows can be infected with PRRSV by exposure of the nasal mucosa or uterine endometrium (Christianson *et al*., 1992; Yaeger *et al*., 1993; Swenson *et al*., 1994; Lager *et al*., 1996). PRRSV is also transmitted by inoculation (Mengeling *et al*., 1994) and presumably by bite wounds. Macrophages within a mucosal surface are the site of primary PRRSV replication, with distribution to regional lymphoid tissue and subsequent systemic distribution to macrophages in multiple tissues and to monocytes (Rossow *et al*., 1995; Rossow *et al*., 1996).

Pulmonary alveolar macrophages (PAMs) from a pig less than 6 weeks old are most susceptible to PRRSV infection, and within a PAM population, PRRSV has a preference for replication within immature macrophages (Choi *et al*., 1994; Mengeling *et al*., 1995).

Following infection viremia rapidly develops and virus can be demonstrated in both serum and monocytes. There is a transient reduction in circulating white cells about four days post-infection and this affects neutrophils and lymphocytes. CD4, CD8 and B cells are all affected in lymph nodes (Christianson *et al*., 1993; Yoon *et al*., 1996). Virus can be demonstrated in serum from the first day after infection and in the lung from them until day 7 post-infection but persists in spleen, tonsil, lymph nodes for linger. Viremia may last for 1-9 days in sows but up to 3-8, or even 12 weeks in young pigs and continues in the presence of antibody. Throughout this period virus is shed in nasal secretions and feces. The virus may penetrate the reproductive tract. In boars the virus may be shed in the semen that some PRRSV-infected boars demonstrate a loss of libido, while most appear clinically unaffected

(Feitsma *et al*., 1992; Yaeger *et al*., 1993; Christopher-Hennings *et al*., 1995; Prieto *et al.*, 1996)). In the sow infection of the embryo can lead to resorption and in fetuses to mummification and stillbirths. Virus is present in lesions in both maternal and fetal placenta. Infection in young piglets leads to destruction of the PAMs rendering the lung susceptible to infection with bacteria such as *P multocida, A.pleuropneumoniae, H.parasuis* and *Chlamydia spp*.. An interstitial pneumonia develops associated with capillary damage. Antibody can be demonstrated from 7-9 days onwards and persists for more than 20 weeks in some animals (Rossow, 1996).

2. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

2.1 Morphology and genomic structures of PRRSV

The disease is caused by the PRRSV, a virus currently classified as a member of the newly established order of *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Meulenberg, 2000). The *Arterivirus* family consists of PRRSV, lactate dehydrogenase-elevating virus (LDV), eqine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Plagemann, 1996; Nelsen *et al*., 1999). PRRSV was isolated in 1991 in the Netherlands (Lelystad virus [LV]) (Wensvoort *et al*., 1991; Nelsen *et al*., 1999) and a virus causing similar symptoms was isolated in the United States in 1992 (VR-2332) (Benfield *et al*., 1992; Collins *et al*., 1992; Nelsen *et al*., 1999). It has been amply demonstrated that a high genetic diversity exists among isolates belonging to the North American type of PRRSV (Meng *et al*., 1995a, b; Kapur *et al*., 1996; Andreyev *et al*., 1997; Gagnon and Dea, 1998; Murtaugh *et al*., 1998; Pirzadeh *et al*., 1998; Wesley *et al*., 1998; Allende *et al*., 1999; Nelson *et al*., 1999; Goldberg *et al*., 2000; Forsberg *et al*., 2002). In contrast, the average genetic diversity of European type of PRRSV has been found to be much lower (Suarez *et al*., 1994, 1996; Drew *et al*., 1997; Le Gall *et al*., 1998). PRRSV has very restricted host specificity. PRRSV grows primarily in porcine alveolar lung macrophages and in macrophages of other tissues (Wensvoort *et al*., 1991; Dea *et al*., 1992; Pol *et al*., 1992; Bautista *et al*., 1993; Wensvoort, 1993; Voicu *et al*., 1994), leading to apoptosis

in secondary cells and immune system suppression (Molitor *et al*., 1997; Murtaugh *et al*., 2002). It was also shown to replicate in testicular germ cells such as spermatids and spermatocytes in infected boars (Sur *et al*., 1997). PRRSV can grow *in vitro* in primary cultures of alveolar lung macrophages and in African green monkey cells or derivatives thereof (CL2621 or MARC-145 cells: (Benfield *et al*., 1992; Bautista *et al*., 1993; Kim *et al*., 1993). Upon transfection of genomic RNA, PRRSV can replicate in several cell lines that cannot be infected by virus particles (Meulenberg *et al*., 1998). PRRSV is thought to enter the host via the standard endocytotic route. Electron microscopy has revealed PRRSV particles present in small vesicles, which appear to be clathrin-coated pits (Kreutz *et al*., 1996). Between 3-6 hours after infection, double membrane vesicles are formed (Pol *et al*., 1992). This is a general feature of an *Arterivirus* infection. These double membrane vesicles are derived from the endoplasmic reticulum (ER) and carry the replication complex (Pedersen *et al*., 1999; Meulenberg, 2000). PRRSV is assembled when prefomed nucleocapsids bud into the lumen of the smooth endoplasmic recticulum or Golgi region or both (Dea *et al*., 1992; Pol and Wagenaar, 1992; Mardassi *et al*., 1994; Dea *et al*., 1995; Dea *et al*., 2000; Meulenberg, 2000). After budding, virions accumulate in vesicles, which move to the plasma membrane where they fuse to release the virus by exocytosis (Dea *et al*., 1995; Pol *et al*., 1997; Dea *et al*., 2000; Meulenberg, 2000). Infected cells become progressively pycnotic and detach from the monolayer two to four days post-infection (p.i.). By indirect immunofluorescence (IIF) and immunoperoxidase, viral antigens can be detected in the perinuclear region as early as 6 h p.i.and the maximum release of PRRSV particles is between 10 to 20 h (Yoon *et al*., 1992; Bautista *et al*., 1993; Kim *et al*., 1993; Dea *et al*., 2000; Meulenberg, 2000). The maximum titers in cell culture are $10^{6.5}$ to $10^{7.5}$ $TCID_{50}$ (50 percent cell culture infective dose/ml) which are usually obtained from clarified cell culture supernatants after 5 to 7 serial passages (Benfield *et al*., 1992; Kim *et al*., 1993; Dea *et al*., 2000; Meulenberg, 2000). The cytopathogenic effect (CPE) observed on both non-porcine cell lines consists of the appearance of small rounded clumps of cells raised above the remainder of the infected monolayer (Benfield *et al*., 1992; Kim *et al*., 1993). On PAMs, the CPE is characterized by the rounding off, clumping, and lysis of cells. Infected cells commonly display a bristling cytoplasmic

membrane and granulation (Wensvoort *et al*., 1991; Dea *et al*., 1992; Dea *et al*., 2000). Destruction of monolayer is usually achieved by 72 to 96 h p.i. (Wensvoort *et al*., 1991; Benfield *et al*., 1992; Kim *et al*., 1993; Mardassi *et al*., 1994; Dea *et al*., 2000).

Purified extracellular virions are pleomorphic, but mostly spherical enveloped particles with a diameter of 45 to 70 nm (Benfield *et al*., 1992; Pol and Wagenaar, 1992; Mardassi *et al*., 1994; Dea *et al*., 2000). Buoyant densities of the infectious viral particles are 1.13 to 1.15 g/ml in sucrose and 1.18 to 1.19 g/ml in cesium chloride (Benfield *et al*., 1992; Wensvoort, 1993; Mardassi *et al*., 1994; Dea *et al*., 2000).

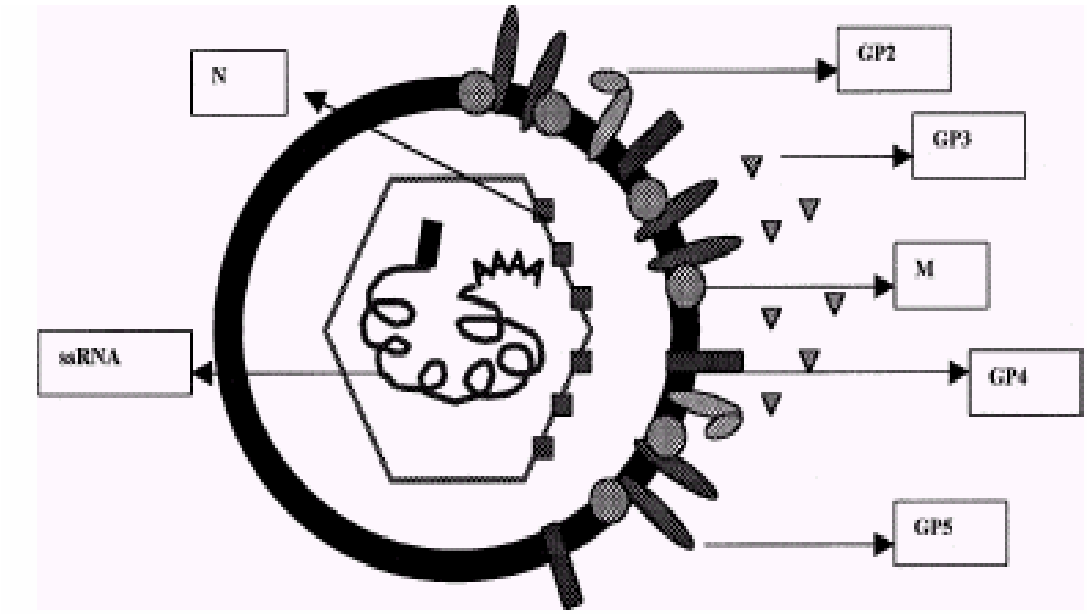


Figure 1 Schematic representation of porcine reproductive and respiratory syndrome virus (Dea *et al.*, 2000).

Table 1 Comparative characteristics of ORFs 2 to 7 products of North American and European strains of PRRSV.

Coding area gene product	Protein	No. of aa residues	
		EU	US
ORF2	GP_2	249	265
ORF3	GP_3	265	254
ORF4	GP_4	183	178
ORF5	GP_5	201	200
ORF6	M[a]	173	174
ORF7	N[a]	128	123

EU European strains; *US* North American strains

[a] In spite of potential glycosylation sites, these proteins are not glycosylated.

Source: a) Meulenberg *et al*., 1993; b) Mardassi *et al*., 1995; c) Meng *et al*., 1995; d) Meulenberg *et al*., 1995; e) Morozov *et al*., 1995; f) Pirzadeh *et al*., 1998; g) Dea *et al*., 2000

PRRSV is 15 kb in length and contains eight ORFs (ORFs 1a, 1b; ORFs 2 to 7) (Figure 1). The virion is mostly spherical in shape, envelope, and possesses a non-segmented single strand positive RNA genome that is encapsidated by the nucleocapsid protein (N) yielding an icosahedral core structure of 20 to 30 nm in diameter (Dea et al., 2000). At least four protein components are envelope-associated: the non-glycosylated matrix protein (M) and the GP_5 glycoproteins which represent major components, whereas GP_2 and GP_4 are two minor components. Both the M and GP_5 are incorporated as disulfide-linked heterodimers. The GP_3 has been also identified as another minor envelope-associated glycoprotein in case of the European LV strain, but in the case of the North American IAF-Klop strain it is rather a soluble and weakly membrane-associated protein (Mardassi *et al*., 1995; Meulenberg *et al*., 1995; Mardassi *et al*., 1996; Meulenberg *et al*., 1996; Meulenberg *et al*., 1997; Gonin *et al*., 1998; Mardassi *et al*., 1998; Dea *et al*., 2000). Comparative characteristics of ORFs 2 to 7 products of PRRSV-EU and PRRSV-US were shown in Table 1.

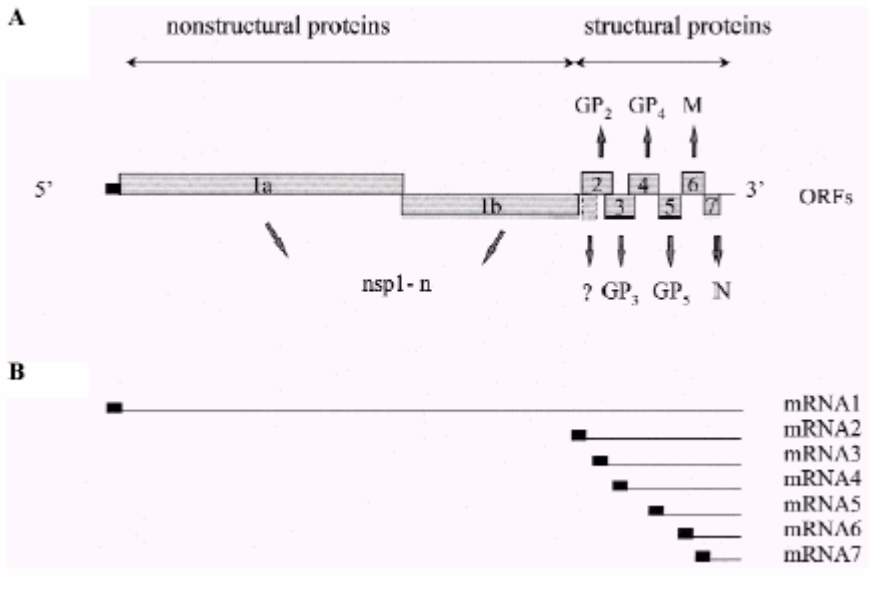


Figure 2 Genome organism of PRRSV (Meulenberg, 2000)

The replicase gene, consisting of the open reading frames (ORFs) 1a and 1b, encodes a polyprotein that is cleaved, forming smaller protein products designated as nonstructural proteins (nsp1-n). ORF 1 is followed by ORFs 2 to 5 encoding glycoproteins GP2 to GP5; ORF 6 encodes the membrane protein (M); and ORF 7 encodes the nucleoprotein (N). An internal ORF present within ORF 2 that might encode an additional structural protein is indicated with dashed lines. 3' nested set of subgenomic mRNAs synthesised during PRRSV replication. The 5' leader, derived from the genomic RNA and fused to the subgenomic RNAs, is shown as a black box (Figure 2).

2.2 Characteristic of ORF7 gene of PRRSV

ORF 7 gene is 372 or 387 in length (for the North American and European strains, respectively). It produces a core made of the 123 or 128 amino acid nucleoprotein (N protein) (for the North American and European strains, respectively). The PRRSV N protein is a small, highly basic protein with an estimated M_r of 14 to 15 kDa (Mardassi *et al*., 1995; Meulenberg *et al*., 1995; Dea *et al*., 2000). The N protein structure is solved to 2.6^{o}A resolution by SAD methods using the

anomalous signal from sulfur. The N protein exists in the crystal as a tight dimmer forming a four-stranded β sheet floor superposed by two long α helices and flanked by two N- and two C-terminal α helices (Doan and Dokland, 2003). The N-terminal half of the N protein contains 26% basic residues (Arg, Lys, and His) that may facilitate its interaction with the RNA genome (Meulenberg *et al*., 1993; Mardassi *et al*., 1994; Meng *et al*., 1995; Meulenberg *et al*., 1995; Dea *et al*., 2000). Contrary to what was previously demonstrated, by constructing mutants to change cysteine residues at positions 23, 75, and 90 of a reference North American strain to proline, and introducing β -breaker mutations at positions 114, 115, and 116, it has been demonstrated that the capsid protein of PRRSV dimerizes through non-covalent interactions. These non disulfide-linked interactions involving the C-terminal domain (specially the last 11 amino acids) of the protein may form the structural basis for viral nucleocapsid formation (Wootton and Yoo, 1999a; Wootton and Yoo, 1999b; Dea *et al*., 2000). Based on the immunoreactivity of N protein using deletion mutants with panels of N-specific MAbs, five domains of antigenic importance have been identified for a reference North American strain, four of them being defined by amino acid 30 to 52, 37 to 52, 69 to 112, and 112 to 123, respectively (Figure 3). Other MAbs revealed the presence of a common conformational antigenic site localized in the central region (amino acid 52 to 69) of the protein (Rodriguez *et al*., 1997; Wootton *et al*., 1998). The amino acids from 37 to 52 are well conserved among isolated of both continents and is the most hydrophilic region of the protein. The 11 most C-terminal amino acids play a critical role in the formation of the conformational epitopes but none of the N-specific MAbs have been found to be associated with virus neutralization (Wootton *et al*., 1998; Dea *et al*., 2000). Four distinct antigenic domains were identified for the reference European LV strain (Meulenberg *et al*., 1998), three of them being defined by the reactivity of anti-N MAbs with linear dodecapeptides whose core sequences consisted of amino acid 2 to 12 (site A), 25 to 30 (site B), and 40 to 46 (site C) (Figure 3). The fourth antigenic domain (site D) is rather conformation-dependent, involving regions between amino acid 51 to 67 and 80 to 90, and was defined by MAbs which did not react with solid-phase dodecapeptides. Site A and C contain epitopes that are conserved in European but not in North American isolated, site B contains epitopes that are conserved in

strains of both continents, and site D contains epitopes that are either conserved or not conserved in the European and North American isolated (Meulenberg *et al*., 1998; Dea *et al*., 2000)

3. *Arteriviridae* family

The family of *Arteriviridae*, which was established in 1996, comprises four enveloped, positive-stranded RNA viruses: lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and porcine reproductive and respiratory syndrome (PRRSV) (Snijder and Meulenberg, 2001). Lactate dehydrogenase-elevating virus (LDV) was discovered by accident in 1960 during a study on the development of methods for early detection of tumors in mice. Equine arteritis virus (EAV) was first isolated in 1957 in Bucyrus, Ohio from lung tissues of an aborted fetus during an epidemic of abortions and arteritis in pregnant mares. Simian hemorrhagic fever virus (SHFV) was first isolated in 1964 during outbreaks of a fatal hemorrhagic fever in macaque colonies in the United States, Russia, and Europe. The virions are spherical, enveloped and 40 to 60 nm in diameter. The virion surface appears smooth and is thought to contain short, if any, spike-like projections. The virion capsid is icosahedral and about 25 to 35 nm in diameter. Buoyant densities of 1.13 to 1.17 g/cm^3 have been reported for *Arterivirus*es in sucrose, and the sedimentation coefficient ranges from 214S to 230S. Virions are fairly stable between pH 6 to 7.5, but are rapidly inactivated at high or low pH. Virions lose their infective quickly when stored at temperatures of 4^{o}C or higher. Virus is efficiently inactivated by lipid solvents and is very sensitive to nonionic detergent treatment (Brinton, 1999; Snijder and Meulenberg, 2001). The genomes of the *Arterivirus*es contain a 3’ poly(A) tract of approximately 50 nucleotides in length and a 5’ type I cap. The length of the genome of EAV is 12.7 kb, that of LDV is 14.1 kb, that of PRRSV is 15.1 kb, and that of SHFV is 15.7 kb.

4. Diagnosis

A diagnosis of PRRS is based on subjective (history, clinical signs, gross and microscopic lesions) and objective (production record analysis, serology, virus detection) factors. PRRS should be considered when there are clinical signs of respiratory disease occurring at any stage of production, when reproductive failure occurs, and when herd performance is suboptimal. Mild or subclinical disease is common, so absence of clinical signs does not ensure a PRRSV-free herd. The development of respiratory disease which is difficult to control in weaners or growers may be associated with PRRS infection. The presence of the disease may also be suspected at post-mortem examination. Anterior lobe lung lesions and the presence of hemorrhages in the carcase may suggest the disease. Histological findings of the presence of an interstitial pneumonia and the absence of alveolar macrophages may also be noted (Taylor, 1995).

4.1 Identification of the agent

Virus isolation is the first and most important step to select the samples most likely to yield useful laboratory information but virological diagnosis of PRRS is difficult. This is mainly because the cell of choice for virus isolation is the porcine alveolar macrophage (PAM), which can be isolated from pigs under 6-8 weeks of age (Mengeling *et al*., 1996a,b). Monkey kidney cell lines (e.g. MARC-145) can be replacement for PAM, but celllines can not support to all isolates, particularly European strains. PAMS can be harvested from swine lungs and macrophages should be validated. Alveolar macrophages *are* seeded in the microtitre plates. After attachment the macrophages are infected with the samples (OIE, 2004). Reverse-transcription Polymerase Chain Reaction (RT-PCR) and mested PCR, that are now commonly used on different tissues including serum, are highly sensitive testes for detecting viral RNA and differentiate North American and European strains of PRRSV (Christopher-Hennings *et al*., 1995; Larochelle and Magar, 1997; Benfield *et al*., 1999; OIE, 2004).

4.2 Serological tests

Serological diagnosis is in general, easy to perform, with specificity and sensitivity, especially on a herd basis. Serology is generally performed with binding assay, such as immunoperoxidase monolayer assay (IPMA) (Wenvoort *et al*., 1991; Drew, 1995), immunofluorescence assay (IFA) (Yoon *et al*., 1992), Serum neutralization assay (SN) (Yoon *et al*., 1994) or the enzyme-linked immunosorbent assay (ELISA) (Albina *et al*., 1992; Cho *et al*., 1996). PRRSV antibodies are detected 7-14 days after infection, reach maximal titers by 30-50 days, and then gradually decline to low or undetectable levels by 4-6 months after infection (OIE, 2004). Neutralising antibodies develop slowly and do not reach high titers. They can be detected from 3 to 4 weeks after infection and they can persist for 1 year or more (Yoon *et al*., 1994)

Immunoperoxidase monolayer assay (IPMA) are detected the antibodies agianst PRRSV. Alveolar macrophages are seed in the wells of microtiter plates. After attachment, the macrophages are infected with PRRSV. The macrophages are fixed and used as a cell substrate for serology. If antibodies are presented in the test serum, they will bind to the antigen in the macrophages. In the next step, The bound antibodies will detected by anti-swine IgG conjugated with horseradish peroxidase (HRPO). The cell substrate is incubated with a substrate solution. Reading of the test is done with an inverted microsope (OIE, 2004).

Immunofluorescence assay (IFA) are used for serum titration purposes. MARC-145 cells are seed in microtiter plates. After attachment, MARC-145 cells are infected with PRRSV. The cells are fixed and used as a cell substrate for serology. If antibodies are presented in the test serum, they will bind to the antigen in MARC-145 cells. In the next step, The bound antibodies will detected by anti-swine IgG conjugated with FITC. Reading of the test is done with an fluorescence microscope (OIE, 2004).

Enzyme-linked immunosorbent assay (ELISA) are used for detect the antibodies agianst PRRSV. The PRRSV antigens are seed in microtiter plates. If antibodies are presented in the test serum, they will bind to the antigen. In the next step, The bound antibodies will detected by anti-swine IgG conjugated with horseradish peroxidase (HRPO). The cell substrate is incubated with a substrate solution. Reading of the test is done with an ELISA reader. ELISA kits are available commercially to setermine the serological status of swine towards PRRSV. Their main advantage is the rapid handling of a large number of samples (OIE, 2004).

5. Prevention and Control

Reducing the clinical and economic effects of PRRSV within infected swine farms is a challenge to veterinarians throughout the world. Despite the high degree of frustration experienced with PRRS since the disease was first reported, progress has been made in the understanding of its epidemiology and in the accuracy of the diagnostic testing procedures.

Therefore, it is critical to routinely isolate and test breeding stock before introducing the infected pigs to a PRRS-negative herd. Replacement stock to be added to naïve herds should be obtained from known negative sources that carry out a regular schedule of herd monitoring. In addition, there should be clear and open communication between veterinarians responsible for the health of the source and recipient herds, respectively, prior to purchase of replacement stock (Benfield *et al*., 1999).

Following the identification of PRRSV and the development of diagnostic tests, practitioners throughout the world have attempted to control the effects of PRRS. The initial attempts were based on the use of strategies known to be effective for controlling other diseases of swine; however, results were inconsistent (Dee *et al*., 1993). The central component of PRRS control is the reduction of the spread of the virus within the breeding herd, thereby preventing the infection of offspring prior to

weaning. Recently, a model for the control of PRRS which focuses on the elimination of subpopulations was developed (Benfield *et al*., 1999).

6. Expression and purification system of recombinant proteins

6.1 *In vitro* expression of recombinant protein

Recombinant DNA technology provides a means of producing large amounts of viral proteins. 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.

Bacterial expression system 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 because it 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 other 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**.**

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). 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 such as moth cell cultures (or caterpillars) may be made to express very large amounts of viral proteins through 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.

Recently, transgenic animal (Echelard, 1996) and plants have attracted attention as combined production hosts and oral vaccine delivery systems (Mason and Arntzen, 1995). Hepatitis B surface antigen (Mason *et a*l., 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).

6.2 *In vitro* expression of recombinant protein in *E. coli*

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 (Appendix Figure D2). 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).

The 11.7 kDa thioredoxin protein is found in yeast, plants, and mammals, as well as in bacteria (Gelhaye *et al*., 2004, Kim *et al*., 2005; Eckenroth *et al*., 2006;). 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 2).

Table 2 Expected molecular weight increasing followed peptide tagging

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

6.3 Purification of recombinant N 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. PRRSV isolates

Eight hundred and eighty eight swine sera collected from the different areas of Thailand were as the source of PRRSV. Sorces of the sera were described in Appendix A. The positive samples for PRRSV was confirmed by Polymerase Chain Reaction (PCR) specific for both the North American (PRRSV-US) and European (PRRSV-EU) strains. The sera stocks were aliquoted in microcentrifuge tubes and stored at -80°c until further studies.

2. Detection of PRRSV positive samples and differentiation between North American and European strains of PRRSV

2.1 Primers

The primers were encompassed on the 3' end of the open reading frames 6 thru the partial sequence of 3'UTR of the PRRSV. The specific primers for the RT-PCR and nested-PCR steps were shown in the table 3. These primers were designed according to the VR-2332 (accession no.AY150564), IAF- EX91 (accession no. L40898), HN1 (accession no.AY457635), IAF-Klop (accession no.U64928), NVSL-14 (accession no.AF396841), NADC-9 (accession no.AF396838), 01NP1.2 (accession no.DQ056373), HB-2(sh)/2002 (accession no.AY262352), LV4.2.1 (accession no. AY588319), AGS-96 (accession no.AF512378), DV (accession no. AF511526) and NL2.2 (accession no.Z92533) isolates of PRRSV.

Table 3 Oligonucleotide primers for the RT-PCR and the nested-PCR steps for the detection and typing of the strain of PRRSV

Primer sequence (5' to 3')	Position in genome	Size of PCR product (bp)	Type detected
External for the One-Step RT-PCR			
C-F: TGTTAAACGGGGAGTGGT C-R: TTGAATAGGTGACTTAGAGGC	EU;14566 – 15022 US;14856 – 15356	EU; 456 US; 500	Common
Internal for the nested PCR			
EU-F: AAAGAAAAGTACAGCTCCGA EU-R: CTGGATGAAAGCGACGCA	14628 – 14884	257	European
US-F: CATCGCTCAGCAAAACCA US-R: CATCATGCTGAGGGTGATGCT	14976 – 15262	287	North American

2.2 RNA Extraction and One-step Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Viral RNA was extracted using acid phenol-guanidinium thiocyanate-chloroform extraction (Chomzcynski, 1987). The RNA templates were subjected to Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique using C-F and C-R primers and SuperScript^{tm} III One-Step RT-PCR System with Platinum$^{®}$ *Taq* DNA Polymerase (Invitrogen, USA) according to the manufacturer instruction. The RT-PCR step was 1 cycle of cDNA synthesis at 45^{o}c for 1 hour and 1 cycle of pre-denaturation at 94^{o}c for 7 minutes and 35 cycles of PCR amplification at 94^{o}c for 45 seconds, 50^{o}c for 1 minute, 72^{o}c for 1 minute and 1 cycle of final extension at 72^{o}c for 15 minutes.

2.3 Differentiation Nested Polymerase Chain Reaction (Nested-PCR)

The PCR products from the RT-PCR step were used as the template for the nested-PCR step. The nested PCR was performed by using 2 pairs of the specific primers which were specific for the European strain (EU-F and EU-R) and the North

American strain (US-F and US-R) (Table 1). The PCR mixture was made up of 100 µl of RT-PCR products, 1.25 mM of $MgCl_2$, 1xbuffer, 0.2 mM of the dNTPs, 1 U of *Taq* DNA polymerase,100 pmol of the primer of each segment. The nested PCR cycle was 1 cycle of pre-denaturation at 94°c for 5 minutes and 35 cycles of PCR amplification at 94°c for 45 seconds, 52°c (European strain) or 56°c (North American strain) for 1 minute, 72°c for 1 minute and 1 cycle of final extension at 72°c for 15 minutes. The amplified nested PCR products were analyzed by electrophoresing 10 µl aliquots through 1.5% agarose gels in TAE buffer [0.04M Tris-acetate (pH8.5), 0.002 M EDTA] for approximately 30 minutes at 100 Volts and gels were stained with EtBr. The electrophoresis results were visualized by UV gel documentation.

3. Cloning for multiple alignment and phylogenetic tree analysis

3.1 Nested-PCR amplification for cloning

Ten PCR products of each the European and North American strains from the RT-PCR step were used as the templates for another nested-PCR amplification. Two pairs of the primers were designed specifically for the ORF7 gene. The forward primer of the European strain was 5' ATGGCCGGTAAAAACCAGAG 3' and the reverse primer was 5' TTAACTTGCACCCTGACTGG 3'. The forward primer for the North American strain was 5' ATGCCAAATAACAACGGCAA 3' and the reverse primer was 5' TCATGCTGAGGGTGATGC 3'. The PCR mixture was made up of 10 µl of RT-PCR products, 1.25 mM of $MgCl_2$, 1xbuffer, 0.2 mM of the dNTPs, 1 U of *Taq* DNA polymerase, 100 pmol of the primer of each segment. The PCR cycle was 1 cycle of pre-denaturation at 94 °c for 5 minutes and 35 cycles of PCR amplification at 94°c for 45 seconds, 55°c (European strain) or 51°c (North American strain) for 1 minute, 72°c for 1 minute and 1 cycle of final extension at 72°c for 15 minutes. The purified PCR products were cloned into pGEM®-T Easy vector (Promega, USA) and the recombinant plasmids were used to transform DH5α competent cells. The positive clones were identified by PCR and restriction endonuclease digestion. The correct recombinant plasmids were subjected to DNA sequencing using dideoxynucleotide sequencing method.

3.2 Sequence alignments and phylogenetic analysis

The nucleotide and deduced amino acid sequences were aligned using the multiple alignment program CLUSTALW (Thompson *et al*., 1994) and compared with the reported sequences in Genbank [LV4.2.1. (European strain prototype) (accession no. AY588319), NL2.2 (accession no.Z92533), DV (accession no. AF511526), AGS-96 (accession no.AF512378), VR-2332 (North American strain prototype) (accession no.AY150564), HB-(sh)/2002 (accession no.AY262352), O1NP1.2 (accession no.DQ056373), NVSL-14 (accession no.AF396841), IAF-EX91 (accession no.L40898), IAF-Klop (accession no.U64928), LDV (accession no. NC001639), EAV (accession no.NC002532) and SHFV (accession no.AF180391)]. The bootstrap option was carried out on 1000 pseudoreplicate data sets to assess the robustness of interior branches of the tree. Phylogenetic analysis of the same alignment was also performed by the maximum parsimony method using the PHYLIP package (Felsinstein, 1989).

4. Cloning for expression N proteins of ORF7 genes of PRRSV

4.1 Competent cells

Genotype of *E.coli* strain TOP10 is 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)

4.2 Construction of pBAD Expression vector

According to phylogenetic study, each selected plasmid pGEM-T contained N gene of the European and the North American strains were used as the template. Primers for amplification of ORF7 genes the European strain were 5'- CAC CAT GGC CGG TAA AAA CCA GAG - 3' (Forward primer) and 5'- ACT TGC ACC CTG ACT GGC G -3' (Reverse primer). Primers for amplification of ORF7 genes of the North American strain were 5'- CAC CAT GCC AAA TAA CAA CGG CAA -3'

(Forward primer) and 5'- TGC TGA GGG TGA TGC TGT - 3' (Reverse primer). The PCR reaction mixtures (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 ORF7-pGEM®-T EASY plasmid) were amplified in the thermocycler (MJ Research, Inc). By the following steps, preheating to 94°c for 5 minutes, amplification by 35 cycles of denaturation at 94°c for 45 seconds, annealing at 52°c (European strain) or 56°c (North American strain) for 1 minute, elongation at 72°c for 1 minute, and finally elongation at 72°c for 10 minutes (Sambrook and Russell, 2001). The amplified products were analysed using agarose gel electrophoresis and used to ligate with plasmid pBAD/D-TOPO vectors (Invitrogen, USA).

4.3 Transformation to competent Top 10 cells

The plasmids pBAD-ORF7 were used to transform into *E. coli* strain TOP 10 (Invitrogen, USA). Transformants were selected on LB medium plates containing kanamycin (50 μg/ml). The selected clones were subcultured and grown overnight and the presence of the N gene was confirmed by PCR.

5. Expression of the N proteins

5.1 Optimal concentration of abrabinose for recombinant N protein expression

The arabinose concentration analysis of the level of protein expression was performed using final concentration of 0.00002, 0.0002, 0.002, 0.02 and 0.2 %. The single colony of transformant carrying ORF7 of each strain was subcultured into 1 ml of LB broth containing 50 μg/ml kanamycin and incubated overnight at 37°c with shaking. Subsequently, 0.1 ml of each overnight cultured was inoculated into 10 ml of pre warmed media with antibiotics, and grown at 37°c with vigorous shaking (approximately 250 rpm) until the OD600 between 0.5-0.7 was achieved. The samples (1 ml) were taken before induction, pelleted (centrifuged at 10,000 rpm for 5 minutes) and resuspended in 80 μl of 1x SDS-PAGE sample buffer and frozen at -

20°c until analyzed by SDS-PAGE. Expression of the recombinant proteins was induced by adding arabinose to a final concentration 0.00002, 0.0002, 0.002, 0.02 and 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 (according to manufacturer recommendation).

5.2 Time-Course analysis of protein expression

The time-course analysis of the level of protein expression was performed by vary induction time from 1-6 hours. The single colony of transformant carrying ORF7 of each strain was subcultured into 1 ml of LB broth containing 50 µg/ml kanamycin and incubated overnight at 37°c with shaking. Thus 0.1 ml of each overnight culture was inoculated into 10 ml of pre-warmed media (with antibiotics), and grown at 37°c with vigorous shaking (approximately 250 rpm) until the OD600 between 0.5-0.7 was achieved. The 1 ml of samples were 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 proteins in *E.coli* were induced by adding arabinose to a final concentration of 0.002 % and then the bacteria were cultured for an additional 8 hours. The samples were collected at 2 hours intervals, pelleted, and resuspended in 80 µl of 1x SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE (according to manufacturer recommendation).

5.3 Growth of standard *E. coli* expression of the recombinant proteins

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°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°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 pellets were then washed with cold PBS and kept at -20^{o}c until used (according to manufacturer recommendation).

5.4 Growth of standard Top10 *E. coli* without pBAD-ORF7 plasmid expression of control *E. coli* protein

The single colony of the Top10 *E. coli* were 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 (approximately 250 rpm) until the OD600 between 0.5-0.7 was achieved (approximately 2-3 hours). Top10 *E. coli* cultures were 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 used (according to manufacturer recommendation).

5.5 Determination of crude recombinant proteins by Dot blot analysis

The crude extracted of *E. coli* containing plasmid pBAD-ORF7 were dotted on nitrocellulose membranes, incubated with 5% skimmed milk for two hours and followed by incubation with 300 µl of 1:10 serum from infected pig 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 membranes were incubated with 300 µl of 1:1000 rabbit anti-swine IgG conjugated with horseradish peroxidase (KPL, USA) for 45 minutes at room temperature. 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**,** USA) for 5-10 min at room temperature (Cochet et al., 1998).

5.6 Analysis of crude recombinant proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The pellets from 1 ml of recombinant Top10 *E.coli* cells were resuspended in 80 µl of 1x SDS-PAGE sample buffer. The crude extracted proteins were analyzed using 12% SDS-PAGE at 120 volts for 60 minutes and stained with Coomassie brilliant blue. For Western blot analysis, proteins on SDS-PAGE were transferred onto nitrocellulose membranes, incubated with 5% skim milk for two hours at 37^{o}c and incubated with 3 ml of 1:10 serum from infected pig for 1 hour at 37^{o}c. After incubation, membranes were washed with PBS (pH 7.4) for three times. Subsequently, the nitrocellulose membranes were incubated with 3 ml of 1:1000 rabbit anti-swine IgG conjugated with horseradish peroxidase (KPL, USA) 45 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 DAB (Sigma, USA) for 5-10 min (Cochet et al., 1998).

6. Purification of the Recombinant Proteins Using Ni-NTA

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

The prepared cell pellets were thawed, resuspended in lysis buffer B (Appendix B) at 5 ml per gram wet weight and sonicated on ice until the lysates become translucence. The suspension was centrifuged at 10,000 rpm for 15 min at 4^{o}c or room temperature to remove the cellular debris (according to manufacturer recommendation) (Qiagen, USA).

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

One millilitre of 50 % Ni-NTA slurry 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 times with 4 ml of washing buffer C (Appendix B). The polyhistidine tag fusion proteins were eluted twice with 0.5 ml elution buffer D and followed by 4 times with 0.5 ml elution buffer E (Appendix B). Each fraction were collected and analyzed by SDS-PAGE (according to manufacturer recommendation) (Qiagen, USA).

6.3 Determination of the recombinant proteins by Dot blot analysis with mouse anti-histidine monoclonal antibody

The crude and purified proteins were dotted on nitrocellulose membrane and incubated with 5% skimmed milk for two hours and followed by incubation with 300 µl of 1:2000 mouse anti-histidine monoclonal antibody (Zymed, USA) for an hours 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 membranes were incubated with 300 µl of 1:300 anti-mouse IgG conjugated with horseradish peroxidase (KPL, USA) for 30 minutes at room temperature. The membranes were washed with PBS for three times and incubated with 300 µl of 0.6 mg/ml DAB (Sigma, USA) for 5-10 min at room temperature (Cochet *et al*., 1998).

6.4 Analysis of the purified recombinant proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with mouse anti-histidine monoclonal antibody

The purified recombinant proteins and proteins extracted from control *E. Coli* were analyzed using 12 % SDS-PAGE at 120 volts for 60 minutes and stained with Coomassie brilliant blue. For Western blot analysis, proteins on SDS-PAGE were transferred on nitrocellulose membranes which were incubated with 5% skim milk and incubated with 3 ml of 1:2000 mouse anti-histidine monoclonal antibody (Zymed, USA) for 1 hour. After incubation, membranes were washed with PBS for three times. Subsequently, the nitrocellulose membranes were incubated with 3 ml of 1:300 anti-mouse IgG conjugated with horseradish peroxidase (KPL, USA) for 45

minutes. The membranes were washed with PBS for three times and incubated with 3 ml of 0.6 mg/ml DAB (Sigma, USA) for 5-10 min (Cochet et al., 1998).

6.5 Analysis of the purified recombinant proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with polyclonal antibodies

The purified recombinant proteins and *E. coli* protein were analyzed using 12% SDS-PAGE at 120 volt for 60 minutes and stained with Coomassie brilliant blue. For Western blot analysis, proteins on SDS-PAGE were transferred on nitrocellulose membranes which were incubated with 5% skim milk and incubated with 3 ml of 1:10 serum from infected pig for 1 hour at 37^{o}c. After incubation, membranes were washed with PBS for three times. Subsequently, the nitrocellulose membranes were incubated with 3 ml of 1:1000 rabbit anti-swine IgG conjugated with horseradish peroxidase (KPL, USA) for 45 minutes at room temperature. The membranes were washed with PBS for three times and incubated with 3 ml of 0.6 mg/ml DAB (Sigma, USA) for 5-10 min (Cochet et al., 1998).

7. Development of an Enzyme-linked immunosorbant assay (ELISA) using purified recombinant nucleoproteins

7.1 Measurement protein concentration by modified Lowry protein assay

The concentrations of purified recombinant proteins and control *E. coli* protein were measured using modified Lowry protein assay kit (Pierce, USA). In brief, 40 µl of each standard and recombinant protein samples were pipetted into each 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. Subsequently, microplate was mixed for 30 seconds and incubated at RT for 30 minutes. The absorbance was

measured at 650 nm using microplate reader (Tecan) (according to manufacturer recommendation)(Pierce, USA).

7.2 Serological assays

In order to determine the PRRSV antibody status of the sera, tests for detection of specific antibodies were undertaken by an indirect ELISA and a commercially available ELISA. The HerdChek Porcine Reproductive and Respiratory Syndrome virus antibody test kit (IDEXX, USA) was used according to the guidelines of the manufacturer.

7.3 Detection antibody against N proteins by Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The microtiter plates were coated with 1, 5 and 10 ng per well of the recombinant nucleoproteins of both strains of PRRSV and 1, 5 and 10 ng per well of the Top 10 *E.coli* as the control for optimal concentration determination. The 5-ng per well coated plates were the optimal concentration and left overnight at 4^{o}C. The plates were then blocked for 1 h at 37^{o}c with 200 µl of 3% BSA per well. Plates were washed three times with 1x PBS-0.05% Tween 20 (pH 7.5). Pig serum were diluted to 1:100 and added 100 µl per well. After addition of the serum, plates were incubated for 90 min at 37^{o}c and then washed three times with 1x PBS-0.05% Tween 20. Subsequently, 100 µl of 1:15,000 goat anti-swine IgG conjugated horseradish peroxidase (Zymed, USA) was added to each well and the plates were incubated for 45 min at 37^{o}c. After washing, two hundred microliters of TMB was added to each well and incubated for 15 -20 min at room temperature. The reaction was stopped by adding 50 µl of stopping solution to each well. The absorbance was measured at the wavelength 620 nm. Each sample was repeated three times. For each serum OD was calculated separately by using the following formular:

% reactivity=100x[(ODsample – ODuntransform *E.coli*) / (ODpositive control – ODnegative control)] (Seuberlich *et al*., 2002).

ODsample was meatured from the average of triplet for each serum sample tested with recombinant proteins. ODuntransform *E.coli* was meatured from the average of triplet for each serum sample tested with untransform TOP10 *E.coli*. ODpositive control was meatured from the average of triplet for the positive serum tested with recombinant proteins and untransform *E.coli*. ODnegative control was meatured from the average of triplet for the negative serum tested with recombinant proteins and untransform *E.coli*.

The sensitivity and specificity of rN I-ELISA were compared with HerdChek ELISA by using the following formular:

Sensitivity (testX) = 100 x number of positives in both X and reference test / total number of positives in reference test.

Specificity (testX) = 100 x number of negatives in both X and reference test / total number of negatives in reference test (Seuberlich *et al*., 2002).

Sensitivity is proportion of subjects classified as `positive' by both the screening and reference tests. Similarly, specificity is proportion of subjects classified as `negative' by both tests (Taylor *et al.*, 1998).

Kappa test calculatation was determined the test agreement by using the following formular:

$$\text{Kappa test} = (OP-EP) / (1-EP)$$

$$OP = (a+d)/n$$

$$EP = [\{(a+b)/n\}x\{(a+c)/n\}]+[\{(c+d)/n\}x\{(b+d)/n\}]$$

a = True positive, b = False positive, c = False negative

d = True negative, n = Number of samples (Pfeiffer, 2002).

RESULTS AND DISCUSSION

Results

1. Development of the Nested RT-PCR assay for differentiation between North American and European strains of PRRSV

By using primers EU-F and EU-R, the amplicons of the European strain were 257 bp and the North American strain was negative (Figure 3A). By using primers US-F and US-R, the amplicons of the North American strain were 287 bp and the European strain was negative (Figure 3B).

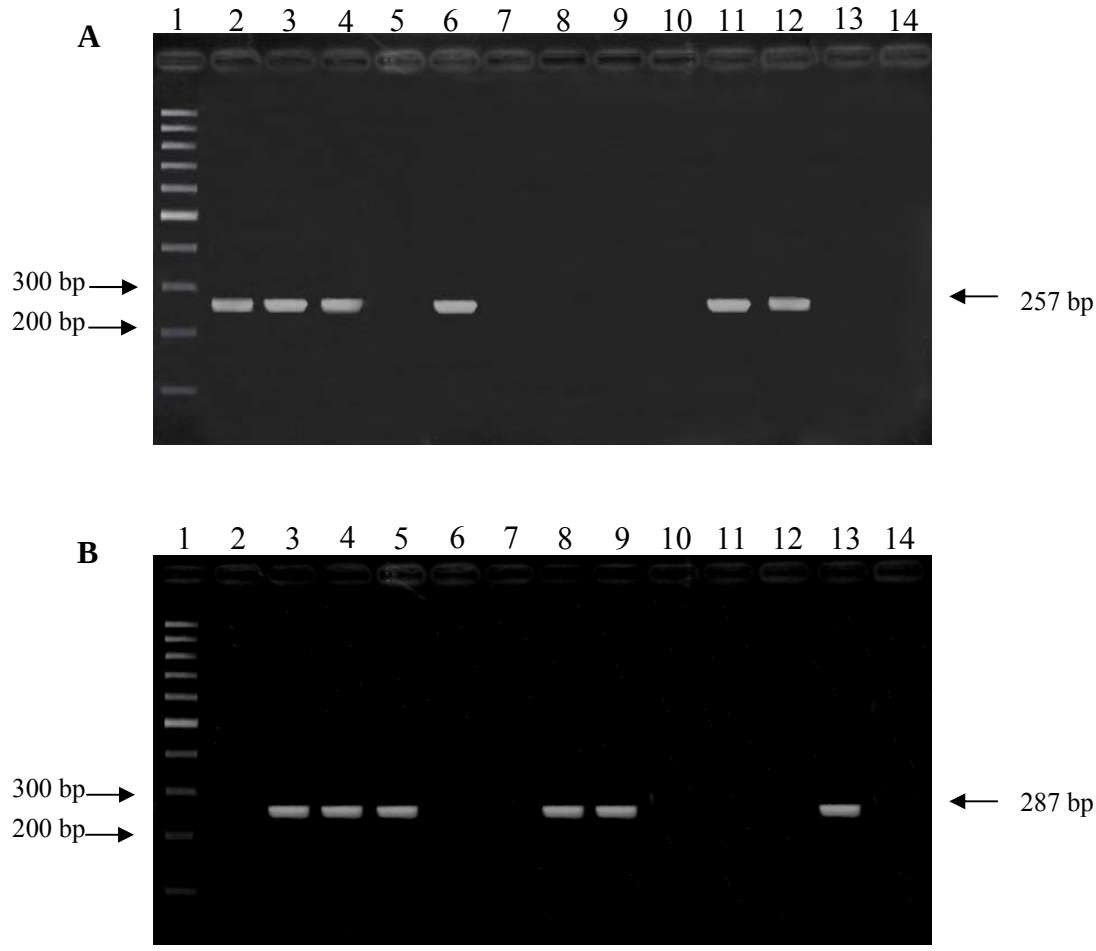


Figure 3 Electrophoresis of the PCR differentiation assay for (A) the European and (B) the North American strain of PRRSV.

(A) Lanes 2, 3. 4, 6 and 11 showed the PCR products of 257 bp. The positive control for the European strain was also 257 bp in size but the positive control for the North American strain was negative.

(B) Lanes 3, 4. 5, 8 and 9 showed the 287 bp. The positive control for the North American strain was also 287 bp in size but the positive control for the European strain was negative. Lane 1 of A and B was 100 bp marker.

2. Phylogenetic study of the ORF7 encoding N protein

2.1 Construction of ORF7 genes of PRRSV

In order to do phylogenetic study of the ORF7 of PRRSVs, the whole ORF7 of both strains was amplified. The amplified products of the whole ORF7 of the European and North American strains were 372 and 387 bp, respectively (Figure 4).

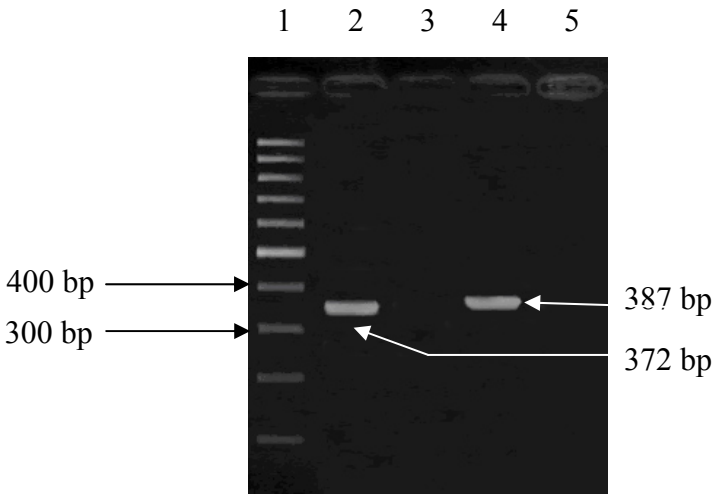


Figure 4 Agarose gel electrophoresis analysis of ORF7 gene of the European and North American strains. The PCR products of the European and North American strains were 372 and 387 bp, respectively.
Lane 1 = DNA marker;
Lane 3 and 5 = negative control;
Lane 2 = PCR product of North American strain;
Lane 4 = PCR product of European strain.

The whole ORF 7 fragments of the European and North American strains were ligated to pGEM-T EASY plasmids. The size of recombinant plasmids contained ORF 7 of the European and North American strains were approximately 3900 and 3400 bp, respectively (Figure 5). The positive colonies were primarily checked for the possession of the recombinant plasmid using PCR assay and digested by restriction

endonuclease. The positive clones of the European and North American strains showed the PCR products approximately 530 and 510 bp, respectively (Figure 5). By digesting with restriction endonuclease *Eco*RI, the positive clones of the European and North American strains had 540 and 520 bp in size, respectively (Figure 5).

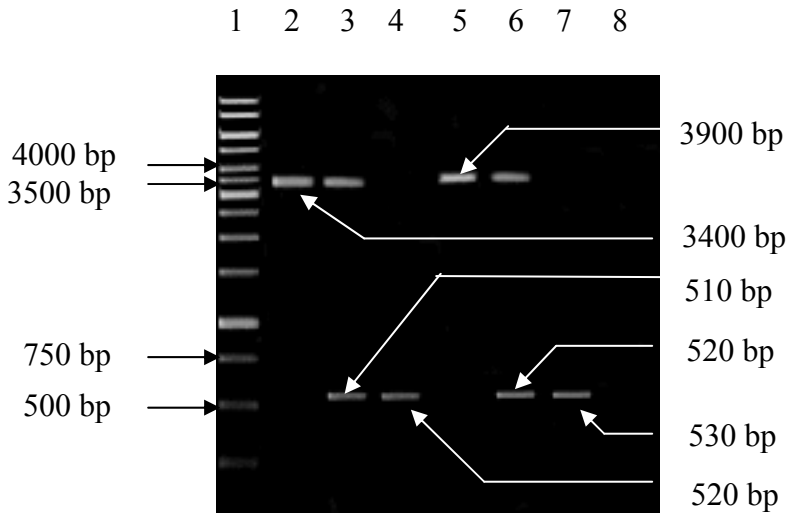


Figure 5 Agarose gel electrophoresis analysis of the PCR results and restriction endonuclease assay of the recombinant plasmid containing the whole ORF7 of the European and North American strains.

Lane 1 = 1 kb marker;

Lane 2 = the recombinant plasmid containing whole ORF7 of PRRSV-US;

Lane 3 = the restriction fragments of the recombinant plasmids containing the whole ORF 7 of PRRSV-US;

Lane 4 = the PCR products of PRRSV-US;

Lane 5 = the recombinant plasmid containing whole ORF7 of PRRSV-EU;

Lane 6 = the restriction fragments of the recombinant plasmids containing the whole ORF 7 of PRRSV-EU;

Lane 7 = the PCR products of PRRSV-EU;

Lane 8 = the negative control**.**

2.2 Sequence alignments and phylogenetic analysis

The sequence comparison of ORF7 of both strains with the reported sequences in Genbank showed homology 98% between the European strains, 95% between the North American strains and 64% between the European and North American strains (Figure 6).

A

```
LV4.2.1     ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAAAGTACAGCTCCGATGGGGAATGGC 60
DV          ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAAAGTACAGCTCCGATGGGGAATGGC 60
NL2.2       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAAAGTACAGCTCCGATGGGGAATGGC 60
KU/N1       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAGTACAGCTCCGATGGGGAATGGC 60
KU/N2       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAGTACAGCTCCGATGGGGAATGGC 60
KU/W1       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
KU/W2       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
KU/S1       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
KU/E1       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
KU/S2       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
KU/NE2      ATGGCCGGTAAAAACNAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
KU/E2       ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
EUvacc      ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
KU/NE1      ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAATACAGCTCCGATGGGGAATGGC 60
AGS-96      ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAGAGTACAGCTCCAATGGGGAATGGC 60
            *************** ****************** * ********* ************

LV4.2.1     CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
DV          CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
NL2.2       CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/N1       CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/N2       CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/W1       CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/W2       CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/S1       CAGCCGGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/E1       CAGCCGGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/S2       CAGCCGGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/NE2      CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/E2       CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
EUvacc      CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
KU/NE1      CAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCGCCAGCAA 120
AGS-96      CAGTCAGTCAATCAACTGTGCCAGTTGCTGGGTGTAATGATAAAGTCCCAGCGCCAGCGA 120
            *** * *************************** ********************** *

LV4.2.1     CCTAGGGGAGGACAGGCCAAAAAGAAAAAGCCTGAGAAGCCACATTTTCCCCTGGCTGCT 180
DV          CCTAGGGGAGGACAGGCCAAAAAGAAAAAGCCTGAGAAGCCACATTTTCCCCTGGCTGCT 180
NL2.2       CCTAGGGGAGGACAGGCCAAAAAGAAAAAGCCTGAGAAGCCACATTTTCCCTTGGCTGCT 180
KU/N1       CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
KU/N2       CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
KU/W1       CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
KU/W2       CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
KU/S1       CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
KU/E1       CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
KU/S2       CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
KU/NE2      CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
KU/E2       CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
EUvacc      CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
```

```
KU/NE1      CCTAGGGGAGGACAGGCAAAAAAAAGAAAGCCTGAGAAGCCACATTTTCCCCTAGCTGCT 180
AGS-96      CCTAAGGGAGGACAGGCCAAAAAGAAAAAGCCTGAGAAGCCACATTTTCCCTTGGCTGCT 180
            **** ************ ***** * ************************* * ******

LV4.2.1     GAAGATGACATCCGGCACCACCTCACCCAGACTGAACGCTCCCTCTGCTTGCAATCGATC 240
DV          GAAGATGACATCCGGCACCACCTCACCCAGACTGAACGCTCCCTCTGCTTGCAATCGATC 240
NL2.2       GAAGATGACATCCGGCACCATCTCACCCAGACTGAACGCTCCCTCTGCTTGCAATCGATC 240
KU/N1       GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
KU/N2       GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
KU/W1       GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCCGCTTGCAATCGATC 240
KU/W2       GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCCGCTTGCAATCGATC 240
KU/S1       GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
KU/E1       GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
KU/S2       GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
KU/NE2      GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
KU/E2       GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
EUvacc      GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
KU/NE1      GAAGATGACATTCGGCACCACCTCACCCAGACCGAACGTTCCCTCTGCTTGCAATCGATC 240
AGS-96      GAAGATGACATTCGGCACCATCTCACCCAAACTGAACGCTCCCTCTGCTTGCAATCGATC 240
            *********** ******** ******** ** ***** ****** **************

LV4.2.1     CAGACGGCTTTCAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
DV          CAGACGGCTTTCAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
NL2.2       CAGACGGCTTTCAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/N1       CAGACGGCTTTTAACCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/N2       CAGACGGCTTTTAACCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/W1       CAGACGGCTTTTAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/W2       CAGACGGCTTTTAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/S1       CAGACGGCTTTTAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/E1       CAGACGGCTTTTAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/S2       CAGACGGCTTTTAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/NE2      CAGACGGCTTTTAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/E2       CAGACGGCTTTTAACCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
EUvacc      CAGACGGCTTTTAACCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT 300
KU/NE1      CAGACGGCTTTTAACCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCGGT 300
AGS-96      CAGACGGCTTTCAATCAAGGCGCAGGAACTGCGTTGCTTTCATCCAGCGGGAAGGTCAGT 300
            *********** ** ****************** ********************** **

LV4.2.1     TTTCAGGTTGAGTTTATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
DV          TTTCAGGTTGAGTTTATGTTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
NL2.2       TTTCAGGTTGAGTTTATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
KU/N1       TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
KU/N2       TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
KU/W1       TTTCAGGTTGAGTTCGTGCTGCCGGTTGCTCATACAGTGCGCCTTATTCGCGTGACTTCT 360
KU/W2       TTTCAGGTTGAGTTCGTGCTGCCGGTTGCTCATACAGTGCGCCTTATTCGCGTGACTTCT 360
KU/S1       TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
KU/E1       TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
KU/S2       TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
KU/NE2      TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
KU/E2       TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
EUvacc      TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
KU/NE1      TTTCAGGTTGAGTTCATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
AGS-96      TTTCAGGTTGAGTTTATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCT 360
            **************  ** ************************* ***************


LV4.2.1     ACATCCGCCAGTCAGGGTGCAAGTTAA 387
DV          ACATCCGCCAGTCAGGGTGCAAGTTAA 387
NL2.2       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/N1       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/N2       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/W1       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/W2       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/S1       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
```

```
KU/E1       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/S2       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/NE2      ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/E2       ACATCCGCCAGTCAGGGTGCAAGTTAA 387
EUvacc      ACATCCGCCAGTCAGGGTGCAAGTTAA 387
KU/NE1      ACATCCGCCAGTCAGGGTGCAAGTTAA 387
AGS-96      ACATCCGCCAGTCAGGGTGCAGCTTAA 387
            **********************  ****
```

B

```
KU/N3       ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAGGGGGGATGGCCAGCCAGTCAAT 60
KU/NE4      ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAGGGGGGATGGCCAGCCAGTCAAT 60
KU/NE3      ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAGGGGGGATGGCCAGCCAGTCAAT 60
KU/S3       ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
VR-2332     ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
01NP1.2     ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
HN1         ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
NADC-9      ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
KU/N4       ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
USvacc      ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
KU/W4       ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
KU/E4       ATGCCAAATAACAACGGCAAGCAGCAAAAGAAAAAGAAAGGGGATGGCCAGCCAGTCAAC 60
NVSL-14     ATGCCAAATAACAACGGCAAGCAGCAAAAGAAAAAGAAGGGGAATGGCCAGCCAGTCAAT 60
HB-2_sh_/200ATGCCAAATAACAACGGCAAGCAGCAAAAGAGAAAGAAGGGGGACGGCCAGCCAGTCAAT 60
IAF-EX91    ATGCCAAATAACAACGGCAGACAGCAAAAGAAAAAGAAAGGGGATGGCCAGCCAGTCAAT 60
IAF-Klop    ATGCCAAATAACAACGGCAAGCAGCAGAAGAAAAAGAAAGGGGATGGCCAGCCAGTCAAT 60
KU/S4       ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAAGGGGGATGGCCAGCCAGTCAAT 60
KU/W3       ATGCCAAATAACAACGGCAAGCAGCAGAAGAGAAAGAGGGGGGATGGCCAGCCAGTCAAT 60
KU/E3       ATGCCAAATAACAACGGCAAGCAGCAAAAGAAAAAGAAAGGGGATGGCCAGCCAGTCAAC 60
            ******************  ***** **** *****  *** * **************

KU/N3       CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
KU/NE4      CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
KU/NE3      CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
KU/S3       CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
VR-2332     CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
01NP1.2     CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
HN1         CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
NADC-9      CAGCTATGTCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
KU/N4       CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
USvacc      CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
KU/W4       CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
KU/E4       CAGCTGTGCCAGATGCTGGGTAAGATCATAGCCCAGCAAAACCAGTCCAGAGGTA-AGGA 119
NVSL-14     CAACTGTGCCAGATGCTGGGTAAGATCATCGCCCAGCAAAACCAGTCCAGAGGCA-AGGG 119
HB-2_sh_/200CAGCTGTGCCAGATGTTGGGTAAGATCATCGCCCAACAAAACCAGTCCAGAGGCA-AGGG 119
IAF-EX91    CAGCTGTGTCAGATGCTGGGTAAAATCATCGCCCAGCAAAACCAGTCCAGAGGTA-AGGG 119
IAF-Klop    CAGCTGTGCCAGATGCTGGGCAGGATCATCGCCCAGCAAAACCAGTCCAGAGGTA-AGGG 119
KU/S4       CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
KU/W3       CAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACCAGTCCAGAGGCA-AGGG 119
KU/E3       CAGCTGTGCCAGATGCTGGGTAAGATCATAGCCCAGCAAAACCAGTCCAGAGGTA-AGGA 119
            ** ** ** ****** **** *  ***** ** ** ***************** * ***

KU/N3       ACCGGGAAAGAAAAATAAGGAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
KU/NE4      ACCGGGAAAGAAAAATAAGGAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
KU/NE3      ACCGGGAAAGAAAAATAAGGAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
KU/S3       ACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
VR-2332     ACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
01NP1.2     ACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
HN1         ACCGGGAAAGAAAATTAAGAAGAAAAACCCGGAGAAGCCCCATTTCCCTCTAGCGACTGA 179
NADC-9      ACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTCCCTCTAGCGACTGA 179
KU/N4       ACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
USvacc      ACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
KU/W4       ACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
```

```
KU/E4          ACCGGGGAAGAAAATAAAGAAGAAGAACCCGGAAAAGCCCCATTTTCCTCTAGCGACTGA 179
NVSL-14        ACCGGGAACGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
HB-2_sh_/200ACCGGGGAAGAAAAATAATAAGAGAAGCCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
IAF-EX91       ACCGGGGAAGAAAAATAAGAAGAAAAACCCGGAAAAGCCCCATTTTCCTCTAGCGACTGA 179
IAF-Klop       ACCGGGGAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACTGA 179
KU/S4          ACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACGGA 179
KU/W3          ACCGGGAAAGAAAAATAAGGAGAAAAACCCGGAGAAGCCCCATTTTCCTCTAGCGACGGA 179
KU/E3          ACCGGGGAAGAAAATTAAGAAGAAGAACCCGGAAAAGCCCCATTTTCCTCTAGCGACGGA 179
               ****** * *****  **  ***  * ****** *********** ******** ** **

KU/N3          AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
KU/NE4         AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
KU/NE3         AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
KU/S3          AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
VR-2332        AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
01NP1.2        AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
HN1            AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
NADC-9         AGATGACGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTATGTCTGTCGTCAATCCA 239
KU/N4          AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
USvacc         AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
KU/W4          AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
KU/E4          AGATGATGTCAGACATCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
NVSL-14        AGATGACGTCAGACATCACTTCACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCGATTCA 239
HB-2_sh_/200AGAAGATGTCAGGCACCACTTCACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCGATCCA 239
IAF-EX91       AGATGACGTCAGACACCACTTTACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
IAF-Klop       AGATGACGTCAGACATCACTTCACCCCTAGTGAGCGGCAATTGTGTCTGTCGTCAATCCA 239
KU/S4          AGACGACGTCAGGCACCATTTCACCCCTGGCGAGCGGCAACTGTGCCTGTCGTCAATTCA 239
KU/W3          AGACGACGTCAGGCACCATTTCACCCCTGGCGAGCGGCAACTGTGCCTGTCGTCAATTCA 239
KU/E3          AGACGACGTCAGGCACCATTTCACCCCTGGCGAGCGGCAACTGTGCCTGTCGTCAATTCA 239
               *** ** ***** ** ** ** ****** * ********* * ** ******** ** **

KU/N3          GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
KU/NE4         GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
KU/NE3         GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
KU/S3          GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
VR-2332        GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
01NP1.2        GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
HN1            GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
NADC-9         GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
KU/N4          GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
USvacc         GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
KU/W4          GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
KU/E4          GACCGCCTTTAATCAAGGCGCTGGGACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
NVSL-14        GACTGCCTTTAATCAAGGCGCTGGAACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
HB-2_sh_/200GACTGCCTTTAACCAGGGCGCCGGGACATGTTCCCTGTCAGATTCAGGGAGGATAAGTTA 299
IAF-EX91       GACAGCCTTTAATCAAGGCGCTGGAACTTGCACCCTGTCAGATTCAGGGAGGATAAGTTA 299
IAF-Klop       GACTGCCTTTAATCAAGGCGCTGGAACTTGTACCCTATCAGATTCAGGGAGAATAAGTTA 299
KU/S4          GACTGCCTTTAATCAAGGTGCTGGAACCTGTACCCTGTCAGATTCGGGGAGGATAAGTTA 299
KU/W3          GACTGCCTTTAATCAAGGTGCTGGAACCTGTACCCTGTCAGATTCGGGGAGGATAAGTTA 299
KU/E3          GACTGCCTTTAATCAAGGTGCTGGAACCTGTACCCTGTCAGATTCGGGGAGGATAAGTTA 299
               *** ******** ** ** ** ** ** **  **** ******** ***** ********


KU/N3          CACTGTGGAGGTTAGTGTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
KU/NE4         CACTGTGGAGCTTAGTGTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
KU/NE3         CACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTACGCCTGATCCGCGTCACAGCATC 359
KU/S3          CACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTGCGCCTGATCCGCGCCACAGCATC 359
VR-2332        CACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
01NP1.2        CACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
HN1            CACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
NADC-9         CACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
KU/N4          CACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTACGCCTGATCCGCGTCACAGCATC 359
USvacc         CACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTACGCCTGATCCGCGTCACAGCATC 359
KU/W4          CACTGTGGAGGTTAGTGTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
```

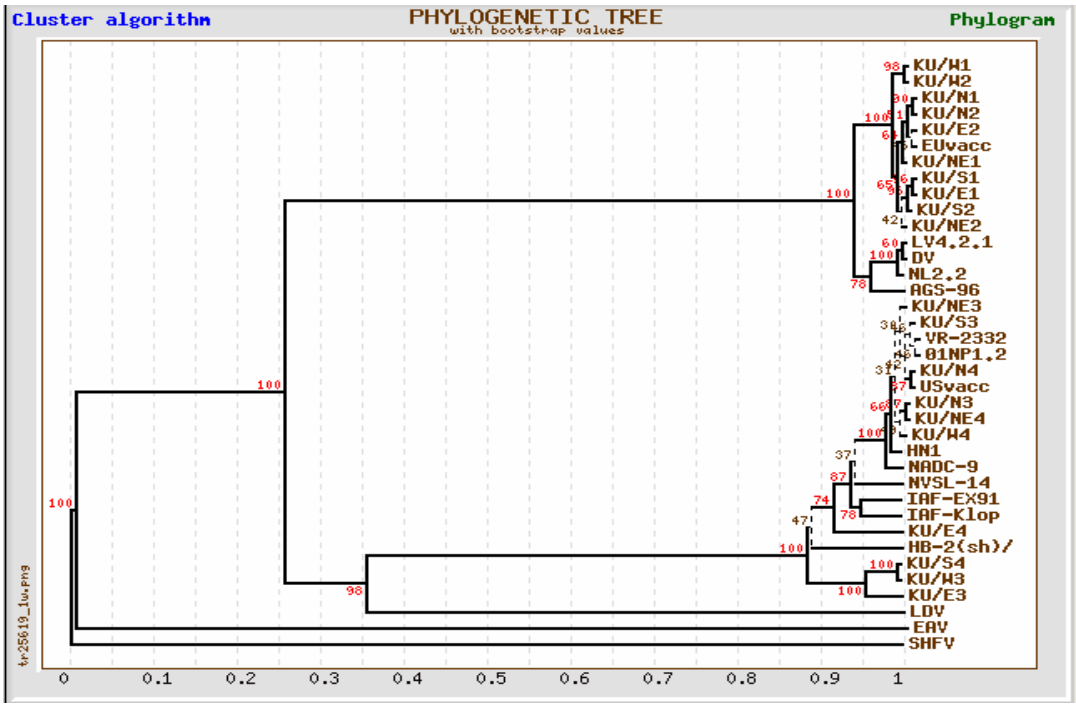
```
KU/E4        CACTGTGGAGGTTAGTGTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
NVSL-14      CACTGTGGAGTTTAGTTTGCCGACGCATCATACTGTGCGCCTGATTCGCGTCACAGCATC 359
HB-2_sh_/200CGCTGTGGAGTTTAGTTTGCCGACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATC 359
IAF-EX91     CGCTGTGGAGTTTAGTTTGCCTACGCATCATACTGTGCGCCTGATTCGCGTCACAGCATC 359
IAF-Klop     CGCTGTGGAGTTTAGTTTGCCTACGCATCATACTGTGCGCCTGATTCGCGTCACAGCATC 359
KU/S4        CGCTGTGGAGTTTAGTTTGCCTACGCATCACACTGTGCGCCTAATTCGCGTCACAGCATC 359
KU/W3        CGCTGTGGAGTTTAGTTTGCCTACGCATCACACTGTGCGCCTAATTCGCGTCACAGCATC 359
KU/E3        CGCTGTGGAGTTTAGTTTGCCTACGCATCACACTGTGCGCCTAATTCGCGTCACAGCATC 359
             * ******** ***** **** ******** ***** ***** ** **** *********

KU/N3        ACCCTCAGCATGA 372
KU/NE4       ACCCTCAGCATGA 372
KU/NE3       ACCCTCAGCATGA 372
KU/S3        ACCCTCAGCATGA 372
VR-2332      ACCCTCAGCATGA 372
01NP1.2      ACCCTCAGCATGA 372
HN1          ACCCTCAGCATGA 372
NADC-9       ACCCTCAGCATGA 372
KU/N4        ACCCTCAGCATGA 372
USvacc       ACCCTCAGCATGA 372
KU/W4        ACCCTCAGCATGA 372
KU/E4        ACCCTCAGCATGA 372
NVSL-14      ACCCTCAGCATGA 372
HB-2_sh_/200ACCCTCAGCATGA 372
IAF-EX91     ACCCTCAGCATGA 372
IAF-Klop     ACCCTCAGCATGA 372
KU/S4        ACCCTCAGCATGA 372
KU/W3        ACCCTCAGCATGA 372
KU/E3        ACCCTCAGCATGA 372
             ************
```

Figure 6 Sequence alignment of ORF7 of both strains comparison to reported ORF7 gene sequences from GenBank using DNASIS and ClustalW program. * = identical nucleotides found in ORF7-pGEM-T EASY plasmids and ORF7 genes from GenBank. (A) Multiple alignments of European strains (B) Multiple alignments of North American strains.

The nucleotide and deduced amino acid sequences of the nucleoprotein gene of PRRSV were used to study phylogeny (Figure 7A and 7B). The pairwise distances between all Thailand isolates of PRRS viruses was more related to LDV than either SHFV or EAV. Moreover, Thailand isolates of the North American strain was more related to LDV than European strain (Figure 7). For Thailand isolates of European strain, the nucleotide sequences were more related to European strain of vaccine than wild types (LV4.2.1,DV ,NL2.2 and AGS-96 strains) (Figure 7A). The amino acid sequences of the European strains isolated form the Northern and Western parts of Thailand were related to LV4.2.1, DV and NL2.2. However, the European strains isolated from other parts of Thailand were more related to European strain of vaccine

(Figure 7B). For North American strain, the most sample nucleotide sequences were more related to VR-2332 strain including North American vaccine but amino acid sequences of most isolates including North American vaccine were related to VR-2332, O1NP1.2, HN1, NADC-9, NVSL-14, IAF-EX91 or IAF-Klop strains except the samples collected from the Eastern part of Thailand (Figure 7A and 7B).

A



B

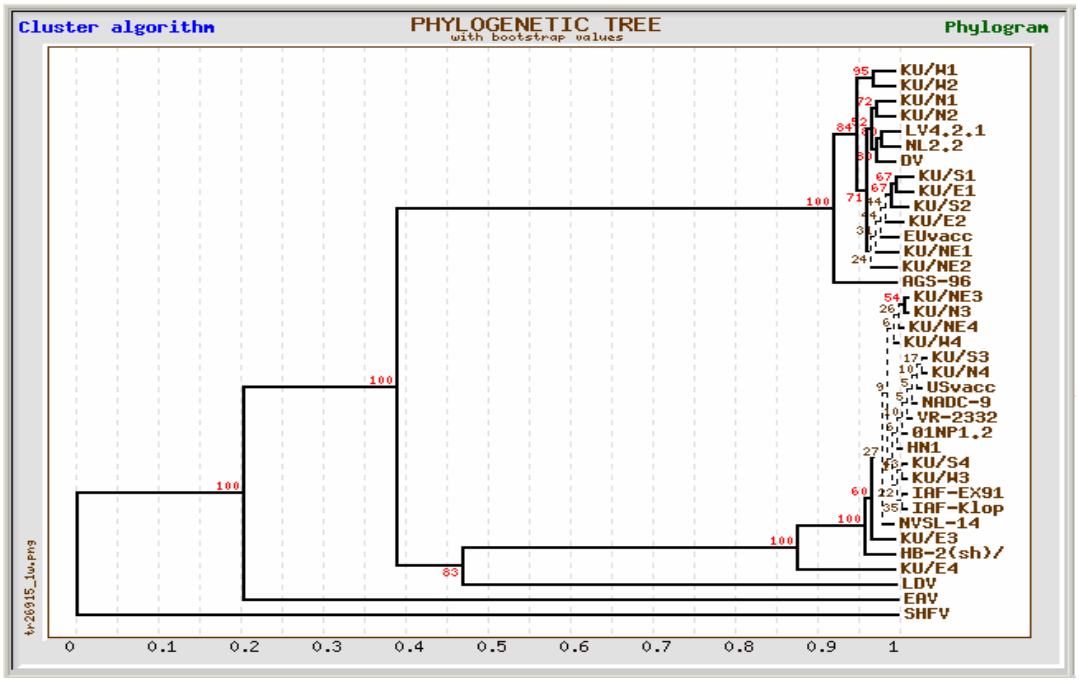


Figure 7 Phylogenetic trees based on (A) nucleotide sequence and (B) amino acid sequence of the European and North American strains of PRRSV inferred from the neighbour-joining method. Numbers on the trees indicate the bootstrap values (percentage) of branches by neighbour-joining (1000 replicates) method.

3. Cloning of N proteins of the European and North American isolates of PRRSV

The ORF7 of both strains were amplified from the ORF7-pGEM-T EASY plasmids contained the selected isolate of both strain PRRSV. The PCR products of the European and North American strains were 387 and 372 bp, respectively (Figure 8).

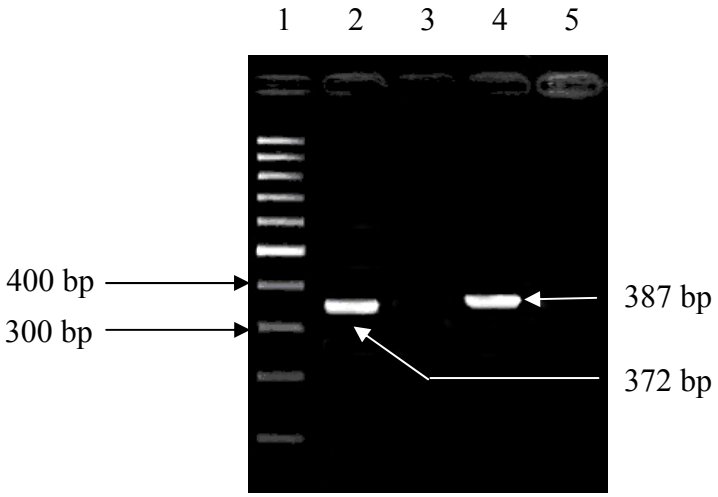


Figure 8 Agarose gel electrophoresis analysis of PCR products of ORF7 from ORF7-pGEM-T EASY plasmids.
Lane 1 = DNA marker;
Lane 2 = PCR product of the ORF7 of PRRSV-US;
Lane 3 = Negative control of PRRSV-US;
Lane 4 = PCR product of the ORF7 of PRRSV-EU;
Lane 5 = Negative control of PRRSV-EU.

The ORF7-pBAD/D-TOPO plasmids of both strains had the size approximately 4800 bp. Subsequently, these plasmids were used to transform *E.coli* strain TOP10. The transformants were primarily checked for the possesion of the recombinant plasmids using PCR assays. The results showed that the selected clones possessed the ORF7 of the North American and European strains which had the size 606 and 622 bp, respectively (Figure 9).

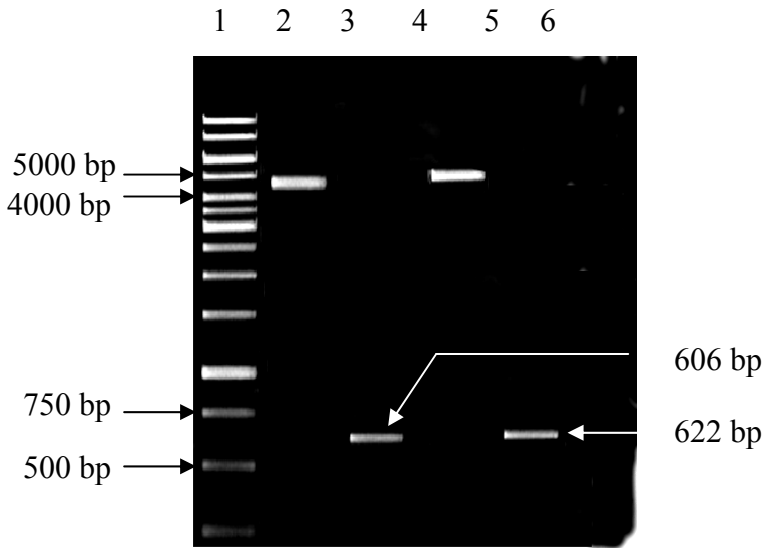


Figure 9 Agarose gel electrophoresis analysis of the PCR products of the selected clone of the transformed *E.coli* strain Top 10.

Lane 1 = DNA marker;

Lane 2 = plasmid contained PRRSV-US ORF7-pBAD/D-TOPO plasmid of the selected clone;

Lane 3 = the PCR product of the selected clone contained PRRSV-US ORF7-pBAD/D-TOPO plasmid;

Lane 4 = plasmid contained PRRSV-EU ORF7-pBAD/D-TOPO plasmid of the selected clone;

Lane 5 = the PCR product of the selected clone contained PRRSV-EU ORF7-pBAD/D-TOPO plasmid;

Lane 6 = negative control.

4. Expression of the recombinant N protein

4.1 Arabinose concentration analysis of protein expression

The different concentrations of arabinose in culture medium were tested for the inducible ability of recombinant N protein expression. The size of the recombinant N proteins was approximately 31 kDa for both strains. By addition of 0.2% to 0.00002% of arabinose, the recombinant N proteins of both strains of PRRSV

were produced whereas the transformants without arabinose showed no induced expression of recombinant N protein (Figure 10 and 11). According to these results, the optimal final concentration of arabinose was 0.002%.

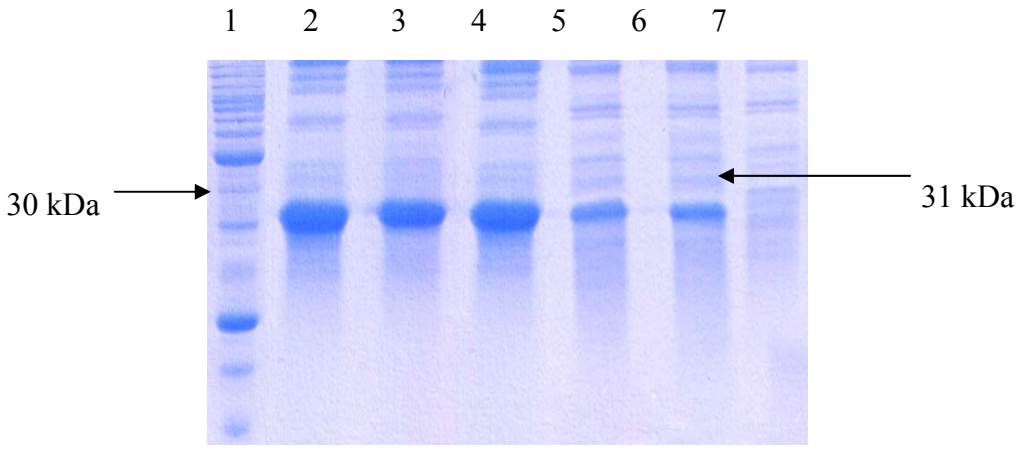


Figure 10 SDS-PAGE analysis of the various concentration of the arabinose for the induction of recombinant N protein of the European strain.
Lane 1 = protein marker;
Lane 2 = induction with 0.2% final concentration of arabinose;
Lane 3 = induction with 0.02% final concentration of arabinose;
Lane 4 = induction with 0.002% final concentration of arabinose;
Lane 5 = induction with 0.0002% final concentration of arabinose;
Lane 6 = induction with 0.00002% final concentration of arabinose;
Lane 7 = crude cells before induction.

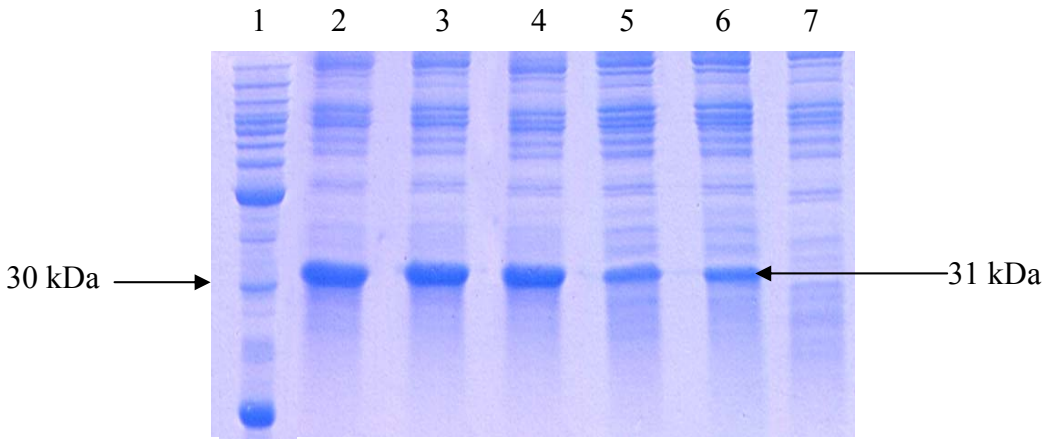


Figure 11 SDS-PAGE analysis of the various concentration of the arabinose for the induction of recombinant N protein of the North American strain.
Lane 1 = protein marker;
Lane 2 = induction with 0.2% final concentration of arabinose;
Lane 3 = induction with 0.02% final concentration of arabinose;
Lane 4 = induction with 0.002% final concentration of arabinose;
Lane 5 = induction with 0.0002% final concentration of arabinose;
Lane 6 = induction with 0.00002% final concentration of arabinose;
Lane 7 = crude cells before induction.

4.2 Time-Course analysis of protein expression

In order to find the optimal time for the expression of the recombinant N proteins, transformants were induced with 0.002% arabinose for 2, 4, 6 or 8 hours. The recombinant N proteins were expressed at 2 hour post-induction and reached the plateau at 4 hours post-induction. According to these results, the optimal induction time was 4 hours (Figure. 12 and 13).

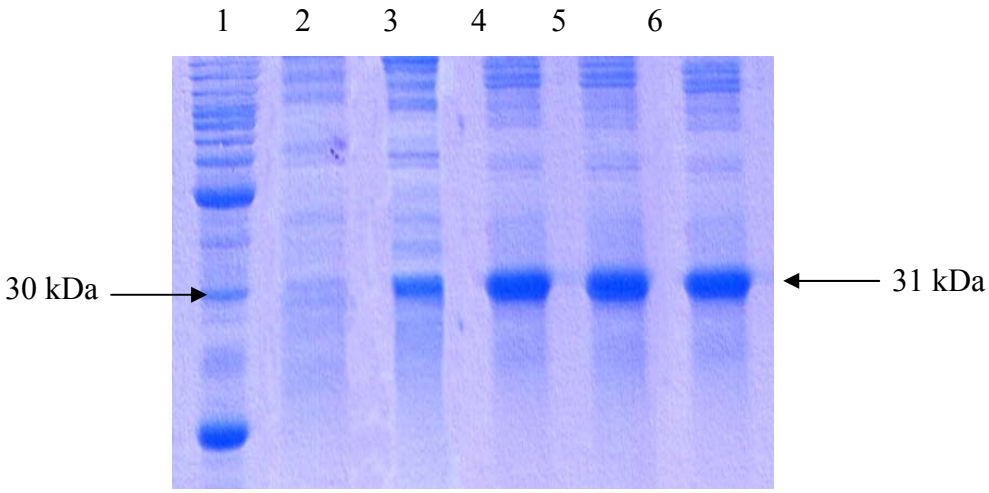


Figure 12 SDS-PAGE analysis of the optimal time for the expression of the recombinant N protein of the European strain.

Lane 1 = protein marker;

Lane 2 = before induction;

Lane 3 = 2 hours post-induction;

Lane 4 = 4 hours post-induction;

Lane 5 = 6 hours post-induction;

Lane 6 = 8 hours post-induction.

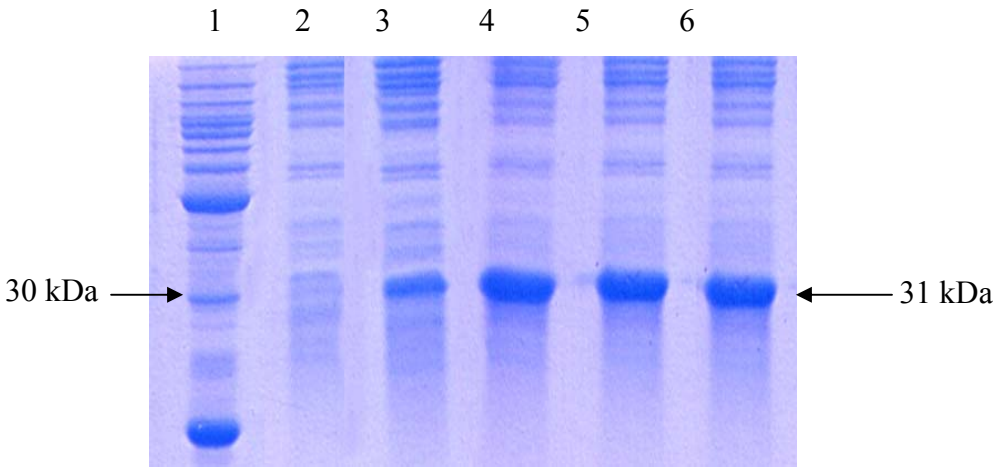


Figure 13 SDS-PAGE analysis of the optimal time for the expression of the recombinant N protein of the North American strain.
Lane 1 = protein marker;
Lane 2 = before induction;
Lane 3 = 2 hours post-induction;
Lane 4 = 4 hours post-induction;
Lane 5 = 6 hours post-induction;
Lane 6 = 8 hours post-induction.

4.3 Standard *E.coli* expression culture

The condition for the expression of the recombinant N protein through out the experiment was 0.002% final concentration arabinose and 4 hours post-induction (Figure 14).

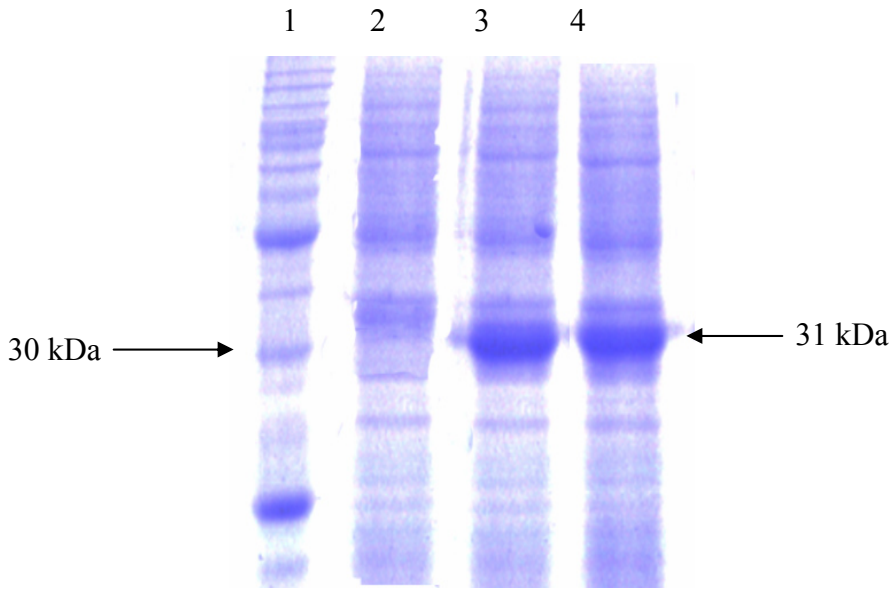


Figure 14 12% SDS-PAGE analysis of the expression of the recombinant N proteins using 0.002% final concentration arabinose and 4 hours incubation.
Lane 1 = protein marker;
Lane 2 = non-induced extract;
Lane 3 = lysate of the recombinant N protein of PRRSV-EU;
Lane 4 = lysate of the recombinant N protein of PRRSV-US.

5. Purification of the recombinant N proteins using Ni-NTA

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

The recombinant N proteins were purified under denaturing condition using the Ni-NTA agarose and were analyzed in SDS-PAGE (Figure 15). 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 6x His tag elute at approximately pH 4.5 (buffer E).

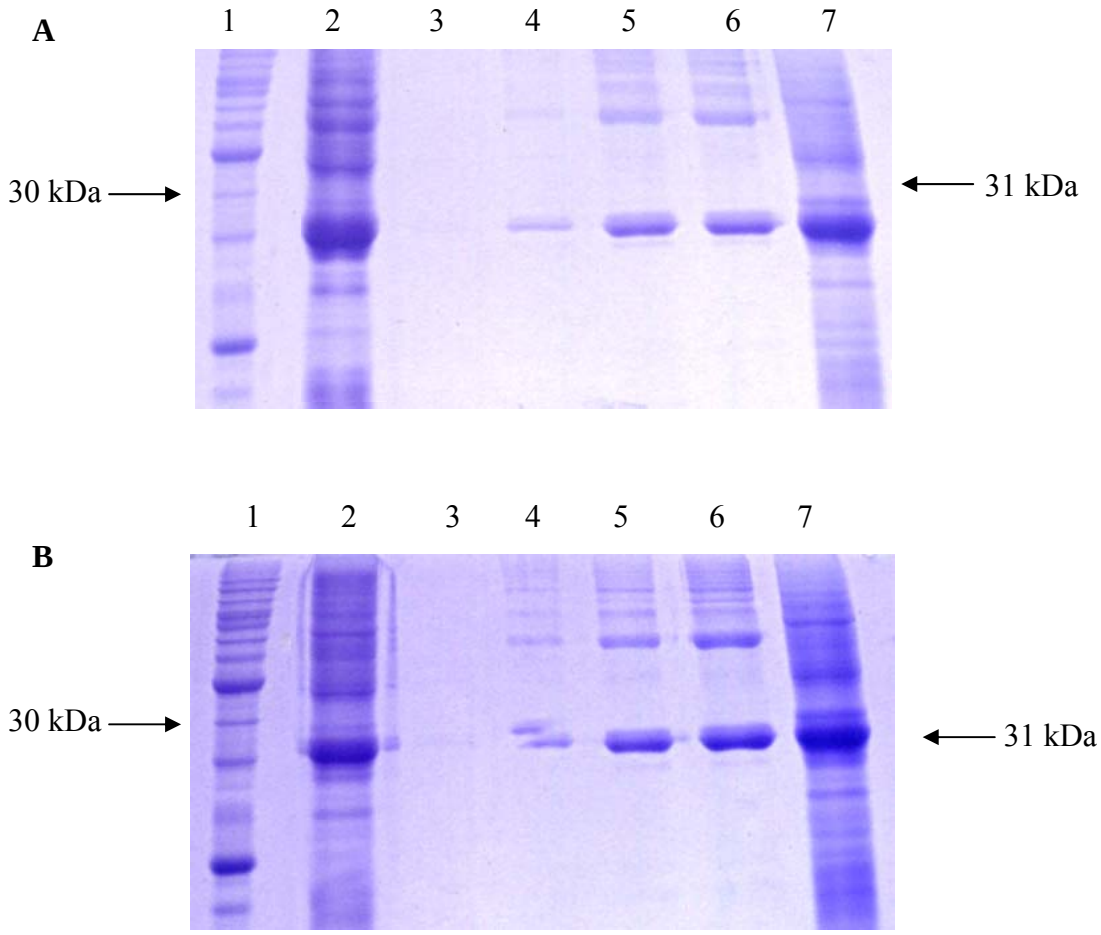


Figure 15 SDS-PAGE analysis of the purified recombinant N proteins of (A) the European and (B) North American strains.

Lane 1 = protein marker;

Lane 2 = crude recombinant N proteins;

Lane 3 = eluted fraction after wash the resin with washing buffer C (pH 6.3);

Lane 4 = eluted fraction from eluted buffer D (pH 5.9);

Lane 5 - 7 = eluted fraction from eluted buffer E (pH 4.5).

5.2 Determination of crude and purified recombinant N proteins by dot blot analysis using anti-histidine monoclonal antibodies

The presence of crude and purified recombinant N proteins was tested with anti-histidine monoclonal antibodies using dot blot analysis. The recombinant N proteins were clearly reacted with anti-histidine monoclonal antibodies. Positive reactions were shown as dark brown spot. The crude and eluted fraction from eluted buffer D and E of recombinant N proteins were stained brown, whereas the protein extracts from normal *E.coli* Top 10 cells and eluted fractions from washing buffer C were negative (Figure 16).

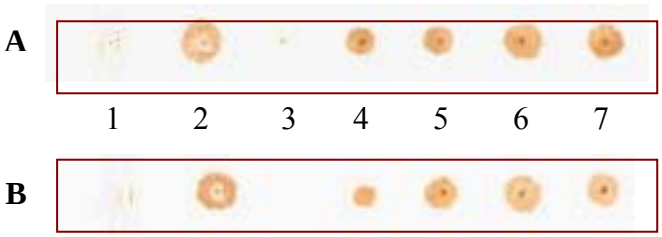


Figure 16 Dot blot analysis of crude and purified recombinant N proteins of (A) European strain and (B) North American strain. Result showed the crude and purified recombinant N proteins (lane 2 and 4-7) clearly react with anti-histidine monoclonal antibodies but not in the normal cell lysates (lane 1) and eluted fractions from washing buffer C (pH 6.3) (lane 3).
Lane 1 = lysates from normal *E.coli* Top 10 cells;
Lane 2 = crude recombinant N proteins;
Lane 3 = eluted fractions from washing buffer C (pH 6.3);
Lane 4 = eluted fractions from eluted buffer D (pH 5.9);L
Lane 5 - 7 = eluted fractions from eluted buffer E (pH 4.5).

5.3 Determination of the recombinant N proteins by Western blot analysis

The Western blot analysis of the purified recombinant N proteins of both strains showed the immunological reaction of anti- histidine monoclonal antibodies (Figure 17) and hyperimmune sera from PRRSV-infected pigs (Figure 18), but not the purified protein from non-transformed *E.coli* strain Top10.

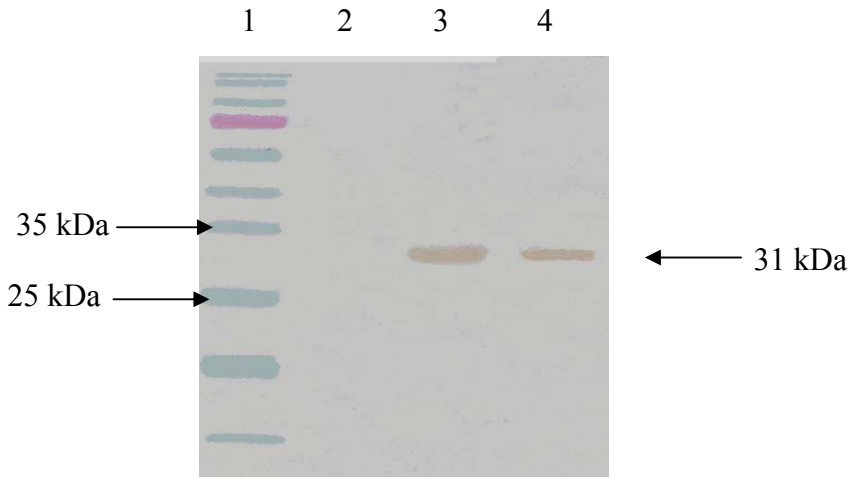


Figure 17 Western blot analysis of the purified recombinant N proteins and protein purified from non-transformed *E.coli* strain Top 10 with the anti-histidine monoclonal antibody.
Lane 1 = protein marker;
Lane 2 = purified protein from non-transformed *E. coli* strain TOP10;
Lane 3 = purified recombinant N protein of PRRSV-EU;
Lane 4 = purified recombinant N protein of PRRSV-US.

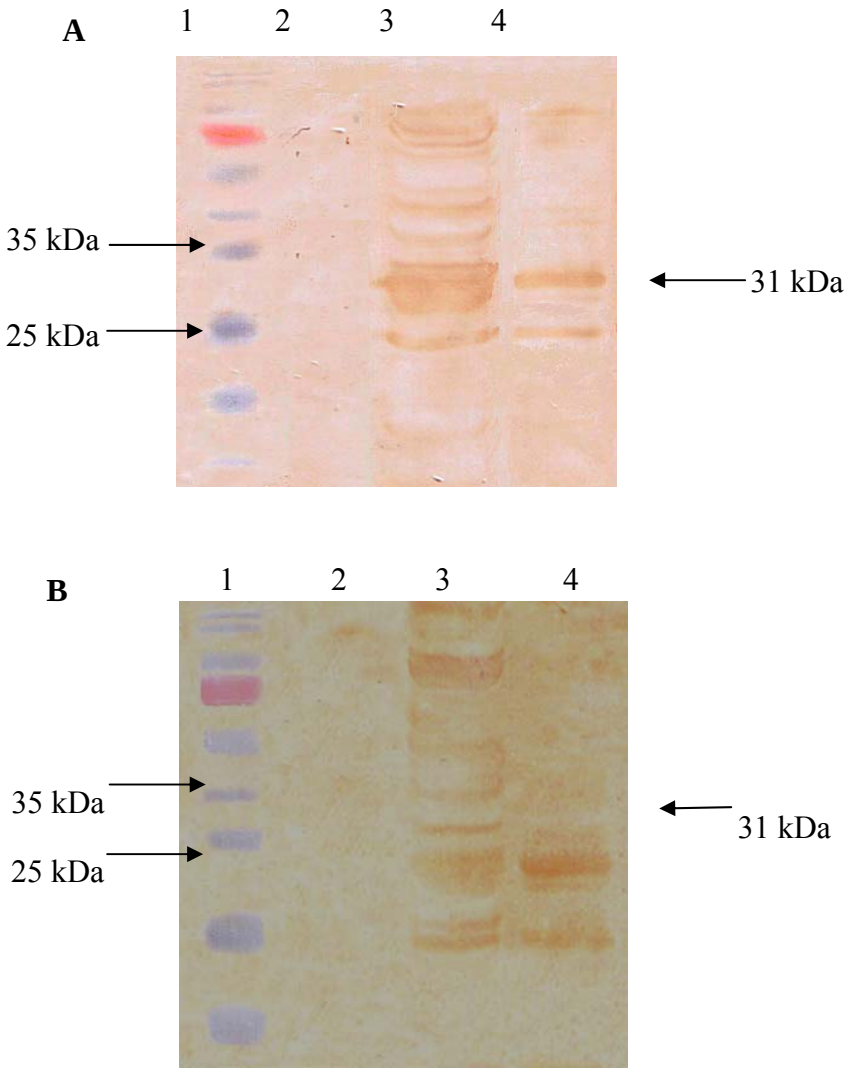


Figure 18 Western blot analysis of the crude and purified recombinant N proteins of (A) European strain, (B) North American strain and protein purified from non-transformed *E.coli* strain Top10 using hyperimmune serum from (A) European strain and (B) North American strain infected pig. Crude recombinant N protein was shown the bands that were the non specific interacted with hyperimmune serum.

Lane 1 = protein marker;

Lane 2 = purified protein from non-transformed *E.coli*;

Lane 3 = crude recombinant N protein;

Lane 4 = purified recombinant N protein.

6. Development of recombinant N proteins (rN proteins) indirect enzyme-linked immunosorbant (I-ELISA)assay

6.1 Indirect ELISA using purified rN proteins as antigen

Two hundred eighty eight swine serum were used for validation of the assay. All serum had already been tested for the negative and positive results by HerdChek ELISA. Two hundred and twenty four sera were positive and sixty four sera were negative. If serum was positive by HerdChek ELISA, the respective serum was considered positive and vice versa for the negative sera. Out of the 288 swine serum, sixty-four negative sera had the reactivity with rN I-ELISA less than 10% reactivity (OD<0.3) when compared to the commercial ELISA. Two hundred and twenty positive sera had the reactivity with rN I-ELISA more than 20% reactivity (OD>0.4). There was a clear separation of the reactivity of the negative and positive sera tested by the rN I-ELISA and had the threshold range in between 10 and 20% reactivity. Hence, serum with reactivity less than 10% reactivity was considered negative and that had the reactivity of more than 20% reactivity was considered positive by rN I-ELISA. Four positive sera had the ambiguous result (within the threshold range) by rN I-ELISA. Ten percentage reactivity was the negative cut-off limit because there were no positive sera reacting below 10% reactivity. (Figure 19).

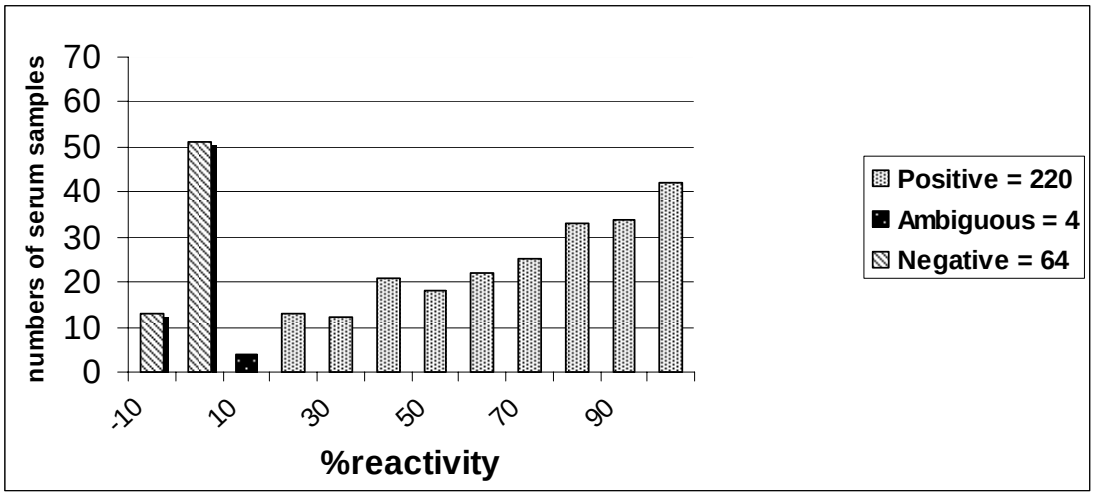


Figure 19 The rN ELISA results of 288 swine sera. There were 64 sera reacted below the negative cut-off value (10% reactivity), 4 sera were reacted within the threshold range (10 - 20% reactivity) and 220 sera were reacted above 20% reactivity. One positive serum by HerdChek ELISA showed ambiguous result by rN I-ELISA.

6.2 Comparison of rN I-ELISA with the commercial ELISA

Total 300 field swine sera were used to compare between rN I-ELISA and HerdChek ELISA (Table 4). Two hundred and fifty one sera were positive by HerdChek ELISA, whereas 249 sera were positive by rN I-ELISA (Table 4). Forty five sera were negative by HerdChek ELISA, whereas 44 sera were negative by rN I-ELISA (Table 4). Four samples showed ambiguous by HerdChek ELISA and 7 sera showed ambiguous by rN I-ELISA. The Kappa value between rN I-ELISA and HerdChek ELISA was 0.913 which indicated the almost perfect agreement between these two tests. When the HerdChek ELISA set as a reference, rN I-ELISA had the sensitivity of 99.2, and the specificity of 97.78 (Table 5).

Table 4 The results of 300 field sera examined by rN I-ELISA and HerdChek ELISA tests

Results	HerdChek ELISA	rN I-ELISA
Positive	251	249
Negative	45	44
Ambiguous	4	7

Table 5 The comparison results of the sensitivity and specificity between the rN I-ELISA and HerdChek ELISA using 300 swine serum samples.

Test X	**Reference test**	
	HerdChek ELISA	
	sensitivity[a]	**specificity**[b]
IDEXX ELISA	-	-
rN I-ELISA	99.2	97.78

[a]Sensitivity (testX) = 100 x number of positives in both X and reference test / total number of positives in reference test.

[b]Specificity (testX) = 100 x number of negatives in both X and reference test / total number of negatives in reference test.

Discussion

1. Differentiation between North American and European strains of PRRSV

The differentiation nested RT-PCR technique for the detection and differentiation of the European and the North American strains of PRRSV were successfully developed. The size of PCR products of the European and North American strains in this study was 257 and 287 bp, respectively, compared with 186 and 107 bp reported previously (Gilbert *et al*., 1997). The main advantage of this assay described herein is the ability to type PRRS virus to one of two genotypes from the samples. The nested assay was sufficiently sensitive that PCR products could be visualized by ethidium bromide staining without the need for additional detection methodology. Combined with a rapid RNA extraction procedure, the RT- nested PCR was user friendly. The higher sensitivity of the nested PCR assay was an advantage in that it permitted direct typing of PRRSV from the porcine serum samples used in this study. The general utility of the nested assay with clinical samples, however, remains to be more thoroughly investigated.

2. Cloning of ORF7 genes for Multiple Alignment and Phylogenetic analysis

The ORF7 genes were successfully cloned to pGEM-T EASY plasmid and were transformed to *E.coli*. The size of the cloned ORF7 gene of the North American and European strains was approximately 510 and 530 bp, respectively. The nucleotide sequences of the recombinant plasmids had 98% homology with the European strain, 95% homology with the North American strain and 64% homology between the European and North American strains. The results of this study were nearly similar to the previous studies (Meng *et al*., 1995b; Suarez *et al*., 1996; Seuberlich *et al*., 2002).

According to the phylogenetic relationships of nucleoprotein gene (ORF7), there were two strains of PRRSV (European and North American strains) in Thailand.

Although applying the very strong bootstrap values, the main branches of these 2 strains were sustained. The Northern and Western Thailand isolates of European strains were more related to wild type virus than those viruses isolated from the other parts of Thailand which more related to European vaccine strain. These results were in agreement with the previous studies in Thailand and Denmark which also showed that the most field isolates of the European strain were more related to European vaccine strain (Thanawongnuwech *et al*., 2004, Stadejek *et al*., 2005). However, all Thailand isolates of North American strains were related to VR-2332 and US strain of vaccine. The studies in Denmark showed that the North American strains in Denmark emerging after the introduction of the North American strain vaccine into Denmark (Stadejek *et al*., 2002, Stadejek *et al*., 2005). According to these results, most PRRSVs circulated in Thailand might be originated from the European and Northern American vaccine strains. The earlier studies also stated that the virus vaccine might mutate become to be the pathogenic virus or the samples were collected at the viraemic period after vaccination (Thanawongnuwech *et al*., 2004, Stadejek *et al*., 2005).

3. Expression of recombinant N proteins in *E.coli*

Dot blot analysis were used to determine structural correctness of the recombinant proteins which has advantages of less time and Ab consumption however the dot blot analysis can not give size of the recombinant proteins whereas Western blot analysis can provide information about the size of the proteins. Using SDS-PAGE and Western blot analysis, the recombinant N proteins expression in *E.coli* were shown as bands of 31 kDa compared with the previous studies that were shown as bands of 15 kDa (Denac *et al*., 1997; Seuberlich *et al*., 2002; Barfoed *et al*., 2004). The 31-kDa recombinant nucleoproteins include 15 kDa of recombinant nucleoproteins and 3 kDa of 6xhis and 13 kDa of thioredoxin from vector. The recombinant N proteins were shown to have interactions with anti-histidine monoclonal antibody and hyperimmune sera from PRRS infected pigs in both assays. This result indicated the recombinant N proteins possess the correct structure.

The yields from expression N protein in *E.coli* were similar but the backgrounds in this study were lower than the previous studies (Seuberlich *et al*., 2002; Barfoed *et al*., 2004). For expresion N protein in baculovirus, specificity with low background was observed in Western blot analysis but the yeild from expression was low level (Denac *et al*., 1997).

4. Detection antibody against PRRSV by recombinant N proteins (rN proteins) indirect enzyme-linked immunosorbant assay (ELISA)

The rN I-ELISA was possible to clearly distinguish the two groups based on their reactivity which allowed to define a threshold range. The selection of a 10% negative cutoff was supported by fact that 96% of the negative sera scored <10% and this value was found to be equally to the commonly used mean (Denac *et al*.,1997). The sera scored negative in IDEXX ELISA and at the same time showed reactivities of > 10% in the rN I-ELISA. The 10%-cutoff value was nearly similar to the some previous studies but the sensitivity and specificity in rN I-ELISA were found to be higher than previous studies (Denac *et al*., 1997; Dea *et al*., 2000b; Seuberlich *et al*.,2002; Ferrin *et al*., 2004). The kappa value of rN I-ELISA was 0.913 that indicated the good agreement when it compared with IDEXX ELISA (Pfeiffer, 2002). One of the major concerns in the current PRRS serodiagnosis is the insufficient sensitivity of the test (Fuch, 1993). In order to reduce the risk of fasle classification, for future diagnostic purposes instead of a single cutoff. In view of the serious economic consequences after positive diagnosis of PRRS, further confirmation tests must be performed in the case of ambiguous or positive results (Denac *et al*., 1997).

CONCLUSION

The differentiation RT-PCR assay for the detection and differentiate of the European and North American strains of PRRSV was successfully developed. According to the phylogenetic relationships of ORF7 gene of PRRSV, there were 2 strains of PRRSV which were the European and the North American strains. Most of the Thailand isolates of PRRSV were closely related to either European or North American strains of vaccine. The ORF7 genes of both strains of PRRSV were expressed and had the size approximately 31 kDa. The rN proteins of both strains of PRRSV were specifically reacting with PRRSV-infected swine serum. The rN I-ELISA was also successfully developed using rN protein. The Kappa values between rN I-ELISA and HerdChek ELISA was 0.913. When the HerdChek ELISA set as a reference, rN I-ELISA had the sensitivity of 99.2, and the specificity of 97.78. When the rN I-ELISA set as a reference, HerdChek ELISA had the sensitivity and specificity of 100. Thus, the recombinant N proteins might be a useful tool for the detection of PRRSV in Thailand.

LITERATURE CITED

Albina, E., Y. Leforban, T. Baron, J.P. Duran and P. Vannier. 1992. An enzyme linked immunosorbent assay (ELISA) for the detection of antibodies to porcine reproductive and respiratory syndrome (PRRS) virus. **Ann. Res. Vet.** 23: 167-176.

Albina. E., P. Vannier, F. Madec, R. Cariolet and J. Torrison. 1994. Persistence of the porcine epidemic abortion and respiratory syndrome (PEARS) virus in infected pigs and farm units**. Proc. Int. Congr. Pig Vet. Soc.** 13: 53.

Allende, R., T.L. Lewis, Z. Lu, D.L. Rock, G.F. Kutish, A. Ali, A.R. Doster and F.A. Osorio. 1999. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. **J. Gen. Virol.** 80: 307-315.

Andreyev, V.G., R.D. Wesley, W.L. Mengeling, A.C. Vorwald and K.M. Lager. 1997. Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field strains based on sequence analysis of open reading frame 5. **Arch. Virol.** 142: 993-1001.

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.

Barfoed, A.M., M.B. Moller, M.H. Jensen, A. Botner and S. Kamstrup. 2004. DNA vaccination of pigs with open reading frame 1-7 of PRRS virus. **Vaccine** 22: 3628-3641

Bautista, E.M., S.M. Goyal, I.J. Yoon, H.S. Joo and J.E. Collins. 1993. Comparison of porcine alveolar macrophages and CL2621 for the detection of porcine reproductive and respiratory syndrome (PRRS) virus and anti-PRRS antibody. **J. Vet. Diagn. Invest.** 5: 163-165.

Benfield, D.A., E.A. Nelson, J.E. Collins, L. Harris, S.M. Goyal, D. Robison, W.T. Christianson, R.B. Morrison, D. Gorcyca and D.W. Chladek. 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332).**J. Vet. Diagn. Invest.** 4: 127-133.

Benfield, D.A., J. Christopher-Hennings, E.A. Nelson, R.R.R. Rowland, J.K. Nelson, C.C.L. Chase, K.D. Rossow and J.E. Collins. 1997. Persistent fetal infection of porcine reproductive and respiratory syndrome (PRRS) virus. **J. Vet. Diagn. Invest.** 1: 455-458.

Benfield, D.A., J.E. Collins, S.A. Dee, P.G. Hallbur, H.S. Joo, K.M. Lager, W.L. Mengeling, M.P. Murtaugh, K.D. Rossow, G.W. Stevenson and J.J. Zimmerman. 1999. Porcine reproductive and respiratory syndrome. In: Disease of swine, pp. 201-232. B.E. Straw, S. D'Allaire, W.L. Mengeling and D.J. Taylor., eds. **Iowa State University Press, Ames**, Iowa, USA.

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

Bilodeau, R., S. Dea, G.P. Martineau and R. Sauvageau. 1991. Porcine reproductive and respiratory syndrome in Quebec. **Vet. Rec.** 129: 102-103.

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

Blaha, T. 1992. Epidemiological investigations into PEARS in Germany: Consequences in fattening pigs. **Proc. Int. Congr. Pig Vet. Soc.** 12: 122-126.

Botner, A., J. Nielsen and V. Bille-Hansen. 1994. Isolation of porcine reproductive and respiratory syndrome (PRRS) virus in Danish swinr herd and experimental infection of pregnant gilts with the virus. **Vet. Microbiol.** 40: 351-360.

Botner, A., B. Strandbygaard, K.J. Sorensen, P. Have, K.G. Madsen, E. S. Madsen and S. Alexandersen. 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. **Vet. Rec.** 141: 497-499.

Brinton, M.A. 1999. *Arterivirus*es (Arteriviridae), pp. 89-97. A. Granoff and R.G. Webster, eds. **Encyclopedia of virology** Vol.1,San Diego:

Cho, H.J., D. Deregt and H.S. Joo. 1996. An ELISA for porcine reproductive and respiratory syndrome: Production of antigen of high quality. **Can J Vet Res.** 60: 89-93.

Choi, C., K. Gustafson, S. Chinsakchai, H. Hill and T. Molitor. 1994. Heterogeneity of porcine alveolar macrophage subpopulations: Immune functions and susceptibility to PEARS virus. **Proc. Int. Congr. Pig Vet. Soc.** 13: 95-97.

Chomzcynski, P.N. 1987. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. **Anal. Biochem.** 162:156-159.

Christianson, W.T., J.E. Collins, D.A. Benfield, L. Harris, D.E. Gorcyca, D.W. Chladek, R.B. Morrison and H.S. Joo. 1992. Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. **Am. J. Vet. Res.** 53: 485-488.

Christianson, W.T., C.S. Choi, J.E. Collins, T.W. Molitor, R.B. Morrison and H.S. Joo. 1993. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and feruses. **Can. J. Vet. Res.** 57: 262-268.

Christopher-Hennings, J., E.A. Nelson, R.J. Hines, J.K. Nelson, S.L. Swenson, J.J. Zimmerman, C.C.L. Chase, M.J. Yaeger and D.A. Benfield. 1995. Persistence of porcine reproductive and respiratory syndrome virus in serum and semen of adult boars. **J. Vet. Diagn. Invest.** 7: 456-464.

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

Collins, J.E., D.A. Benfield, W.T. Christianson, L. Harris, J.C. Hennings, D.P. Shaw, S.M. Goyal, S. McCullough, R.B. Morrison, H.S. Joo, D. Gorcyca and D. Chladek. 1992. Isolation of swine infertility respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. **J. Vet. Diagn. Invest.** 4: 117-126.

De Vries, A.A.F., E.D. Chirnside, M.C. Horzinek and P.J.M. Rottier. 1992. Structural proteins of equine arteritis virus. **J. Virol.** 66: 6294-6303.

Dea, S., R. Bilodeau, R. Sauvageau and G.P. Martineau. 1990. Virus isolations from farms in Quebec experiencing severe outbreaks of respiratory and reproductive problems. **J. Virol.** 15: 67-72.

Dea, S., R. Bilodeau, R. Athanassious, R. Sauvageau and G.P. Martineau. 1992. Swine reproductive and respiratory syndrome virus in Quebec: isolation of an enveloped virus serologically-related to Lelystad virus. **Can. Vet. J.** 33: 801-808.

Dea, S., N. Sawyer, R. Alain and R. Athanassious. 1995. Ultrastructural characteristics and morphogenesis of porcine reproductive and respiratory syndrome virus propagated in the highly permissive MARC-145 cell clone. **Adv. Exp. Med. Biol.** 380: 95-98.

Dea, S., C.A. Gagnon, H. Mardassi, B. Pirzadeh and D. Rogan. 2000a. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison on the North American and European isolates. **Arch. Virol.** 145: 659-688.

Dea, S., L. Wilson, D. Therrien and E. Cornaglia. 2000b. Competitive ELISA for detection of antibodies to porcine reprotductive and respiratory syndrome virus using recombinant E.coli-expressed nucleocapsid protein as antigen**. J. Virol. Method** 87: 109-122.

Dee, S.A., R.B. Morrison and H.S. Joo. 1993. Eradication of PRRS virus using multi-site production and nursery depopulation. **Swine Heal. Prod.** 1: 20-23.

Dee, S.A. and H.S. Joo. 1994. Clinical investigation of resurrent reproductive failure associated with PRRS virus infection in a sow herd. **J. Am. Vet. Med. Assoc.** 205: 1017-1018.

Denac, H., C. Moser, J.D. Tratschin and M.A. Hofmann. 1997. An indirect ELISA for the detection of antibodies against porcine reproductive and respiratory syndrome virus using recombinant nucleocapsid protein as antigen. **J. Virol. Methods** 65: 169-181.

Doan, D.N.P and T. Dokland. 2003. Structure of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus. **Structure** 11: 1445-1451.

Drew, T.W. 1995. Comparative serology of porcine reproductive and respiratory syndrome in eight European laboratories, using immunoperoxidase monolayer assay and enzyme-linked immunosorbent assay. **Rev. Sci. Tech. Off. Inta. Epiz..** 14: 761-775.

Drew, T.W., J. P. Lowings and F. Yapp. 1997. Variation in open reading frames 3, 4 and 7 among porcine reproductive and respiratory syndrome virus isolates in the UK. **Vet. Microbiol.** 55: 209-221.

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

Eckenroth, B., K. Harris, A.A. Turanov, V.N. Gladyshev, R.T. Raines and R.J. Hondal. 2006. Semisynthesis and characterization of mammalian thioredoxin reductase. **Biochemistry** 45: 5158-5170.

Epperson, B. and L. Holler. 1997. An abortion storm and sow mortality syndrome. **Can. Vet. J.** 9:479-484.

Faaberg, K.S and P.G.W. Plagemann. 1995. The envelope proteins of lactate dehydrogenase-elevating virus and their membrane topography. **Virology** 21: 512-525.

Feitsma, H., H.J. Frooten and F.W. Schie. 1992. The effect of porcine epidemic abortion and respiratory syndrome (PEARS) on sperm production. **Proc. Int. Congr. Anim. Reprod.** 12: 1710-1712.

Felsenstein, J. 1989. PHYLIP: Phylogeny inference package (Version 3.2). **Cladistics** 5. 164-166.

Ferrin, N., Y. Fang, C. Johnson, M. Murtaugh, D. Polson, M. Torremorell, M. Gramer and E. Nelson. 2004. Validation of a blocking enzyme-linked immunosorbent assay for detection of antibodies against porcine reproductive and respiratory syndrome virus. **Clin. Diag. Lab. Immuno.** 11: 503-514.

Forsberg, R., T. Storgaard, H.S. Nielsen, M.B. Oleksiewicz, P. Cordioli, G. Sala, J. Hein and A. Botner. 2002. The genetic diversity of European type PRRSV is similar to that of the north American type but is geographically skewed within Europe. **Virology** 299: 38-47.

Fuch, A. 1993. PRRS. **Can. Vet. J.** 32:78-83.

Gagnon, C.A and S. Dea. 1998. Differentiation between porcine reproductive and respiratory syndrome virus isolate by restriction fragment length polymorphism of their ORFs 6 and 7 genes. **Can. J. Vet. Res.** 62: 110-116.

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

Gelhaye, E., N. Rouhier, J. Gerard, Y. Jolivet, J. Gualberto, N. Navrot, P.I. Ohlsson, G. Wingsle, M. Hirasawa, D.B. Knaff, H. Wang, P. Dizengremel, Y. Meyer and J.P. Jacquot. 2004. A specific form of thioredoxin h occurs in plant mitochondria and regulates the alternative oxidase. **Proc. Natl. Acad. Sci.** 101: 14545-14550.

Gilbert, S.A., R. Larochelle, R. Magar, H.J. Cho and D. Deregy. 1997. Typing of porcine reproductive and respiratory syndrome viruses by a multiplex PCR assay. **J. Clin. Microbiol.** 35: 264-267.

Goldberg, T.L., E.C. Hajn, R.M. Weigel and G. Scherba. 2000. Genetic geographical and temporal variation of porcine reproductive and respiratory syndrome virus in Illinois. **J. Gen. Virol.** 81: 171-179.

Gonin, P., H. Mardassi, C.A. Gagnon, B. Massie and S. Dea. 1998. A nonstructural and antigenic glycoprotein is encoded by ORF 3 ofthe IAF-Klop strain of porcine reproductive and respiratory syndrome virus. **Arch. Virol.** 143: 1927-1940.

Gordon, S. C. 1992. Effects of blue-eared pig disease on a breeding and fattening unit. **Vet. Rec.** 130: 513-514.

Goyal, S.M. 1993. Porcine reproductive and respiratory syndrome. **J. Vet. Diagn. Invest.** 5: 656-664

Halbur, P.G., P.S. Paul, X.J. Meng, M.A. Lum, J.J. Andrews and J.A. Rathje. 1996. Comparative pathogenicity of nine U.S. porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesare-an-derived, colostrum-deprived pig model. **J. Vet. Diagn. Invest.** 8: 11-20.

Halbur, P.G. and E. Bush. 1997. Update on abortion storms and sow mortality. **Swine Heal. Prod.** 5: 73.

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.

Hesse,R.A., L.P. Couture, M.L. Lau S.K. Dimmick and S.R. Ellsworth. 1996. Efficacy of Prime Pac PRRS in controlling PRRS reproductive disease Homologous challenge. **Proc. Annu. Meet. Am. Assoc. Swine Pract.** 2:103-105.

Hopper, S.A., M.E. White and N. Twiddy. 1992. An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. **Vet. Rec.** 131: 140-144.

Indik, S., L. Valı´cek, D. Klein and J. Kla´nova´. 2000. Variations in the major envelope glycoprotein GP5 of Chech strains of porcine reproductive and respiratory syndrome virus. **J. Gen. Virol.** 81: 2497–2502.

Invitrogen. 2004. pBAD Directional TOPO Expression Kits: Instruction manual, p. 1-42.

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

Kapur, V., M.R. Elam, T.M. Pawlovich and M.P. Murtaugh. 1996. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the Midwestern United States. **J. Gen. Virol.** 77: 1271-1276.

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.

Keffaber, K.K. 1989. Reproductive failure of unknown etiology. **ASSP. Newsletter.** 1: 1-9.

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

Kim, H.S., J. Kwang, I.J. Yoon, H.S. Joo and M.L. Frey. 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. **Arch. Virol.** 133: 477-483.

Kim, J.A., P. Sunjoo, K. Kangwha, R.S. Goo and K.S. Won. 2005. Activity assay of mammalian 2-cys peroxiredoxins using yeast thioredoxin reductase system. **Ana. Biochemistry** 338: 216-223.

Kono, Y., T. Kanno, M. Shimizu, S. Yamada, S. Ohashi, M. Nakamine and J. Shirai. 1996. Nested PCR for detection and typing of porcine reproductive and respiratory syndrome (PRRS) virus in pigs. **J. Vet. Med. Sci.** 58: 941-946.

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.

Kreutz, L.C. and M.R. Ackermann. 1996. Porcine reproductive and respiratory syndrome virus enters cells through a low pH dependent endocytic pathway. **Virus Res.** 42: 137-147.

Kuwahara, H., T. Nunoya, M. Tajima, A. Kato and T. Samejima. 1994. An outbreak of porcine reproductive and respiratory syndrome in Japan. **J. Vet. Med. Sci.** 56: 901-909.

Lager, K.M., W.L. Mengeling and S.L. Brockmeier. 1996. Effect of post-coital intrauterine inoculation of porcine reproductive and respiratory syndrome virus on conception in gilts. **Vet. Rec.** 138: 227-228.

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.

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.

Le Gall, A., O. Legeay, H. Bourhy, C. Arnauld, E. Albina and A. Jestin. 1998. Molecular variation in the nucleoprotein gene (ORF 7) of the porcine reproductive and respiratory syndrome virus (PRRSV). **Virus Res.** 54: 9-21.

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.

Lindhaus, W. and B. Lindhaus. 1991. Tatselhafte Sweinekrankheit. **Praktische Tierarztl.** 25: 423-425.

Loemba, H.D., S. Mounir I, H. Mardassi, D. Archambault z, and S. Dea. 1996. Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. **Arch. Virol.** 141:751-761.

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.

Madsen, K.G., C.M. Hansen, E.S. Madsen, B. Strandbygaard, A. Botner and K.J. Sorensen. 1998. Sequence analysis of porcine reproductive and respiaratory syndromevirus of the American type collected from Danish swine herds. **Arch. Virol.** 143: 1683-1700.

Mardassi, H., R. Athanassious, S. Mounir and S. Dea. 1994a. Porcine reproductive and respiratory syndrome virus: morphological, biochemical and serological characteristics of Quebec isolates associated with acute and chronic outbreaks of porcine reproductive and respiratory syndrome. **Can. J. Vet Res.** 58: 55-64.

Mardassi, H., S. Mounir and S. Dea. 1994b. Identification of major differences in the nucleocapsid protein genes of a Quebec strain and European strains of porcine reproductive and respiratory syndrome virus. **J. Gen. Virol.** 75: 681-685.

Mardassi, H., S. Mounir and S. Dea. 1995. Molecular analysis of the ORF 3-7 of porcine reproductive and respiratory syndrome virus, Quebec reference strain. **Arch. Virol.** 140: 1405-1418.

Mardassi, H., S. Mounir and S. Dea. 1996. Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. **Virology** 221: 98-112.

Mardassi, H., P. Gonin, C.A. Gagnon, B. Massie and S. Dea. 1998. A subset of porcine reproductive and respiratory syndrome virus (PRRSV) GP3 glycoprotein is released into the culture medium of cells as a non-virion-associated and membrane-free (soluble) form. **J. Virol.** 72: 6298-6306.

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.

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.

Meng, X.J., P.S. Paul, P.G. Halbur and I. Morozov. 1995a. Sequence comparison of open reading frame 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. **J. Gen. Virol.** 76: 3181-3188.

Meng, X.J., P.S. Paul, P.G. Halbur and M.A. Lum. 1995b. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): Implication for the existence of two genotypes of PRRSV in the USA and Europe. **Arch. Virol.** 140: 745-755.

Mengeling, W.L., K.M.Lager and A.S. Vorwald. 1994. Temporal characterization of transplacental infection of porcine fetuses with PRRS virus. **Am J Vet Res.** 55: 1391-1398.

Mengeling, W.L., K.M.Lager and A.S. Vorwald. 1995. Diagnosis of porcine reproductive and respiratory syndrome. **J. Vet. Diagn. Invest.** 7: 3-16.

Mengeling, W.L., K.M.Lager, A.S. Vorwald, R.D. Wesley and D.F. Clouser. 1997. An update of research at the National Animal Disease Center on current strains of porcine reproductive and respiratory syndrome (PRRS) virus. **Proc. Allen. D. Leman. Swine Conf.** 24: 138-145.

Meredith, M.J. 1992. Review of porcine reproductive and respiratory syndrome, pp. 1-24. **Pig Disease Information Centre**. Cambridge, U.K.

Meredith, M.J. 1995. Porcine reproductive and respiratory syndrome (PRRS), pp. 5-10. **Pig Disease Information Centre**. Cambridge, U.K.

Meulenberg, J.J.M., M. Hulst, E.J. De Meijer, P.L.J. Moonen, A. Petersen-Den Besten, E.P. De Kluyver, G. Wensvoort and R.J.M. Moormann. 1993. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. **Virology** 192: 62-72.

Meulenberg, J.J.M., A. Petersen-Den Besten, E.P. De Kluyver, R.J.M. Moormann, W.M.M. Schaaper and G. Wenvoort. 1995. Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. **Virology** 206: 155-163.

Meulenberg, J.J.M and A. Petersen-Den Besten. 1996. Identification and characterization of a sixth structural protein of Lelystad Virus: the glycoprotein GP2 encoded by ORF 2 is incorporated in virus particle. **Virology** 225: 44-51.

Meulenberg, J.J.M., A.P. van Nieuwstadt, A. van Essen-Zandbergen and J.P.M. Langeveld. 1997. Post- translational processing and identification of a neutralization domain of the GP4 protein encoded by ORF 4 of Lelystad virus. **J. Virol.** 71: 6061-6067.

Meulenberg, J.J.M., J.N.A. Bos-de Ruijter, G. Wensvoort and R.J.M. Moormann. 1998. Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. **J. Virol.** 72: 380-387.

Meulenberg, J.J.M. 2000. PRRSV, the virus. **Vet. Res.** 31: 11-21.

Molitor, T.W., J. Xiao and C.S. Choi. 1996. PRRS virus infection of macrophages: Regulation by maturation and activation state. **Proc. Annu. Meet. Am. Assoc. Swine. Pract.** 27: 563-569.

Molitor, T.W., E.M. Bautista and C.S. Choi. 1997. Immunity to PRRS: double-edged sword. **Vet. Microbiol.** 55: 265-276.

Morozov, I., X.J. Meng and P.S. Paul. 1995. Sequence analysis of open reading frames (ORFs) 2 to 4 of a US isolate of porcine reproductive and respiratory syndrome virus. **Arch. Virol.** 140: 1313-1319.

Morrison, R.B., J.E. Collins, L. Harris, D.W. Chladek, D.E. Gorcyca, H.S. Joo, W. Christianson, D.A. Benfield, W.E. Marsh, S. Goyal and J.F. Annelli. 1992. Sero-epidemiologic investigation of porcine epidemic abortion and respiratory syndrome (PEARS, PRRS, SIRS). **Proceeding of International Congress of Pig Veterinary Society** 12:114.

Murakami, Y., A. Kato, T. Tsuda, T. Morozumi, Y. Miura and T. Sugimura. 1994. Isolation and serological characterization of porcine reproductive and respiratory syndrome (PRRS) viruses from pigs with reproductive and respiratory disorders in Japan. ***J. Vet. Med. Sci.*** 56: 891-894.

Murtaugh, M.P., K.S. Faaberg, S. Yuan and V. Kapur. 1997. Interrelatedness of PRRS virus isolates in North America, pp. 146-149. **In Proceeding of 24th Allen K. Leman Conference**, N.Y.

Murtaugh, M.P., K.S. Faaberg, J. Laber, M. Elam and V. Kapur. 1998. Genetic variation in the PRRS virus. **Adv. Exp. Med. Biol.** 440: 787-794.

Murtaugh, M.P., Z. Xiao and F. Zuckermann. 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. **Viral. Immunol.** 15: 533-547.

Nelsen, C.J., M.P. Murtaugh and K.S. Faaberg. 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. **J. Virol.** 73: 270-280.

OIE. 2004. Porcine reproductive and respiratory syndrome. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Available Sorce: http://www.oie.int/eng/OIE/organisation/en_LR.htm, July 23, 04.

Pedersen, K.W., Y. van der Meer, N. Roos and E.J. Snijder. 1999. Open reading frame 1a-encoded subunits of the *Arterivirus* replicase induce endoplasmic recticulum-derived double-membrane vesicles which carry the viral replication complex. **J. Virol.** 73: 2016-2026.

Pesch, S., C. Meyer and V.F. Ohlinger. 2005. New insights into the genetic diversity of European porcin reproductive and respiratory syndrime virus (PRRSV). **Vet. Micro.** 107: 31-48.

Pfeiffer, D.U. 2002. Kappa test, pp. 38-39. **Veterinary Epidemiology: an introduction**. Royal Veterinary Collage, Hertfordshire, UK..

Pirzadeh, B., C.A. Gagnon and S. Dea. 1998. Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus major envelope GP5 glycoprotein. **Can. J. Vet. Res.** 62: 170-177.

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

Plagemann, P.G.W. 1996. Lactate dehydrogenase-elevating virus, pp. 1105-1120. D.M. Knipe and P.M. Howley, eds. **Fields virology**, Philadelphia.

Pol, J.M., J.E. van-Dijk, G. Wensvoort and C. Terpstra. 1991. Pathological, ultrastructural, and immunogistochemical changes caused by Lelystad virus in experimentally induced infections of mystery swine disease. **Vet. Q.** 13: 137-143.

Pol, J.M. and F. Wagenaar. 1992. Morphogenesis of Lelystad virus in porcine lung alveolar macrophages. **Am. Assoc. Swine Prat. Newslett.** 4: 29.

Pol, J.M.A., F. Wagenaar and J.E.G. Reus. 1997. Comparative morphogenesis of three PRRS virus strains. **Vet. Microbiol.** 55: 203-208.

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

Prieto, C., P. Suarez, J.M. Bautesta, R. Sanchez, S.M. Rillo, I. Simarro, A. Solana and J.M. Castro. 1996. Semen changes in boars after experimental infection with porcine reproductive ande respiratory syndrome (PRRS) virus. **Theriogenology** 45: 383-395.

Rodriguez, M.J., J. Sarraseca, J. Jarcia, A. Sanz, J. Plana- Duran and I. Casal. 1997. Epitope mapping of the nucleocapsid protein of European and North American isolates of porcine reproductive and respiratory syndrome virus. **J. Gen. Virol.** 78: 2269-2278.

Rossow, K.D., J.E. Collins, S.M. Goyal, E.A. Nelson, J. Christopher-Hennings and D.A. Benfield. 1995. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. **Vet Pathol.** 32: 361-373.

Rossow, K.D., D.A. Benfield, S.M. Goyal, E.A. Nelson, J. Christopher-Hennings and J.E. Collins. 1996. Chronological immunohistochemical detection and localization of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. **Vet Pathol.** 33: 551-556.

Sambrook, J., E.F. Fritsch and T. maniatis. 1989. Molicular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, USA.

Sambrook, J. and D.W. Russell. 2001. Transformation, pp. 320-325. **Molecular Cloning: A Laboratory Manual.** Spring Harbor Laboratory Press, N. Y.

Sarah, K. and D. Yoo. 2003. Homo-Oligomerization of the porcine reprotductive and reapiratory syndrome virus nucleocapsid protein and the role of disulfide linkages. **J Virol.** 77: 4546-4557.

Schleif, R. 1992. DNA looping. **Ann. Rev. Biochem**. 61: 199-223.

Seuberlich, T., J.D. Tratschin, B. Thur and M.A. Hofmann. 2002. Nucleocapsid protein-based enzyme-linked immunosorbent assay for detection and differentiation of antibodies against European and North American porcine reproductive and respiratory syndrome virus. **Clin. Dign. Labor. Immunol.** 9: 1183-1191.

Shimizu, M., S. Ymada, K. Kawashima,S. Ohashi, S. Shimizu and T. Ogawa. 1996. Changes of lymphocyte subpopulations in pig infected with pocine reproductive and respiratory syndrome (PRRS) virus. **Vet. Immunol. Immunopathol.** 50: 19-27.

Sirinarumitr, T., Y. Zhang, J.P. Kluge, P.G. Halbur and P.S. Paul. 1998. A pneumo-virulent United States isolate of porcine reproductive and respiratory syndrome virus induces apoptosis in bystander cells both in vitro and in vivo. **J. Gen. Virol.** 79: 2989-2995.

Snijder, E.J., H. van Tol, K.W. Pedersen, et al. 1999. Identification of a novel structural protein of *Arterivirus*es. **J. Virol.** 73: 6335-6345.

Snijder, E.J and J.J.M. Meulenberg. 2001. *Arterivirus*es, pp. 1205-1220. In D.M. Knipe and P.M. Howley, eds. **Fields Virology** Vol.1, Philadephia..

Solano, G., J. Segales and C. Pijoan. 1995. Interaction between porcine reproductive and respiratory syndrome virus (PRRSV) and *Haemophilus parasui*, p. 21. **In Proceeding of 2nd International Symposium on Porcine Reproductive and Respiratory Syndrome**, Copenhagen, Denmark.

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

Stadejek, T., A. Stankevicius, T. Storgaard, M.B. Oleksiewicz, S. Belak, T.W Drew and Z. Pejsak. 2002. Identification of radically different variants of porcine reproductive and respiratory syndrome virus in Eastern Europe: towards a common ancestor for European and American viruses. **J. Gen. Virol.**83: 1861-1873.

Stadejek, T., M. Porowski and Z. Pejsak. 2005. Viraemia and seroconversion in piglets following vaccination with PRRSV-EU type vaccine – a field observation. **Bull. Vet. Inst. Pulawy**. 49: 273-277,

Stevenson, G.W., W.G. Van-Alstine and C.L. Kanitz. 1994. Characterization of infection with endemic porcine reproductive and respiratory syndrome virus in swine gerd. **J. Am. Vet. Med. Assoc.** 204: 1938-1942.

Suarez, P., R. Zardoya, C. Prieto, A. Solana, E. Tabares, J.M. Bautista and J.M. Castro. 1994. Direct detection of the porcine reproductive and respiratory syndrome (PRRS) virus by reverse Polymerase Chain Reaction (RT-PCR). **Arch. Virol.** 135: 89-99.

Suarez, P., R. Zardoya, M.J. Martin, C. Prieto, J. Dopazo, A. Solana and J.M. Castro. 1996. Phylogenetic relationships of European strains of porcine reproductive and respiratory syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. **Virus Res.** 42: 159-165.

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

Sur, J.H., A.R. Doster, J.S. Christian, J.A. Galeota, R.W. Wills, J.J. Zimmermann and F.A. Osorio. 1997. Porcine reproductive and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis, and induces germ cell death by apoptosis. **J. Virol.** 71: 9170-9179.

Swenson, S.L., H.T. Hill, J.J. Zimmerman, L.E. Evans, R.W. Wills, K.J. Yoon, K.J. Schwartz, G.C. Althouse, M.J. McGinley and A.K. Brevik. 1994. Artificial in semination of gilts with porcine reproductive and respiratory syndrome (PRRS) virus-contaminated semen. **Am Assoc Swine Pract Newsl.** 2: 19-23.

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.

Taylor, D.J. 1995. The Porcine Reproductive and Respiratory Syndrome (PRRS), pp. 58-64. **Pig diseases**. Cambridge, U.K..

Thanawongnuwech, R., A. Amonsin, A. Tatsanakit and S. Damrongwatanapokin. 2004. Genetics and geographical variation of porcine reproductive and respiratory syndrome virus (PRRSV) in Thailand. **Vet. Micro.** 101: 9-21.

Terpstra, C., G. Wensvoort and J.M. Pol. 1991. Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. **Vet. Q.** 13: 131-136.

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.

Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. **Nucleic Acids Res.** 22, 4673-4680.

Torrison, J., M. Knoll and B. Wiseman. 1996. Evidence of pig-to-pig transmission of modified-live PRRS virus vaccine. **Proceeding of American Association of Swine Practitioner**s: 89-91.

Van Alstine, W.G., G.W. Stevenson and C.L. Kanitz. 1996. Porcine reproductive and respiratory syndrome virus does not exacerbate *Mycoplasma hyopneumoniae* infection in young pigs. **Vet Microbiol.** 49: 297-303.

Van Nieuwstadt, A.P., J.J.M. Meulenberg and A. van Essen-Zandbergen. 1996. Proteins encoded by open reading frames 3 and 4 of the genome of Lelystad virus (Arteriviridae) are structural proteins of the virion. **J. Virol.** 70: 4767-4772.

Van Woensel, P., J. Van Der Wouw and N. Visser. 1994. Detection of porcine reproductive and respiratory syndrome virus by poltmerase chain reaction. **J. Virol. Methods.** 47: 273-278.

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

Voicu, I.L., A. Sllim, M. Morin and M. A.S.Y. Elazhary. 1994. Interaction of porcine reproductive and respiratory syndrome virus with swine monocytes. **Vet. Rec.** 134: 422-423.

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.

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., C. Terpstra, J.M.A. Pol, E.A. Ter Laak, M. Bloemraad, E.P. De Kluyver, C. Kragten, L. Van Buiten, A. Den Besten, F. Wagenaar, J.M. Broekhuilsen, P.L.J.M. Moonen, T. Zetstra, E.A. De Boer, H.J. Tibben, M.F. De Jog, P. Van't Veld, G.J.R. Groenland, J.A. Van Gennep, M.T. Voets, J.H.M. Verheijden andJ. Braamskamp. 1991. Mystery swine disease in the Netherlands: the isolation of Lelystad virus. **Vet. Q.** 13: 121-130.

Wensvoort, G. 1993. Lelystad virus and the porcine epidemic abortion and respiratory syndrome. **Vet Res.** 24: 117-124.

Wesley, R.D., W. L. Mengeling, V. Andreyev and K. M. Lager. 1996. Differentiation of vaccine (Strain RespPRRS) and field strains of porcine reproductive and respiratory syndrome virus by restriction enzyme analysis, pp. 141-143. **In Proceeding of 27th American Association of Swine Practitioners**, Iowa.

Wesley, R.D., W.L. Mengeling, K.M. Lager, D.F. Clouser, J.G. Landgraf and M.L. Frey. 1998. Differentiation of a porcine reproductive and respiratory syndrome virus vaccine strain from North American field strains by restriction fragment length polymorphism analysis of ORF 5. **J. Vet. Diagn. Invest.** 10: 140-144.

White, M. 1991. Blue ear disease of pigs. **Vet. Rec.** 128: 574.

White, M.E.C. 1992. The clinical signs and symptoms of blue-eared pig disease (PRRS). **Pig Vet. J.** 28: 62-68.

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.

Wootton, S.K., E.A. Nelson and D. Yoo. 1998. Antigenic structure of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus. **Clin. Diagn. Lab. Immunol.** 5: 773-779.

Wootton, S.K and D. Yoo. 1999a. Homotypic interactions of the capsid protein of porcine reproductive and respiratory syndrome virus, p. 146. **Proceedings of the 18th Annual Meeting of the American Society for Virology**, University of Massachusetts, Amherst.

Wootton., S.K and D. Yoo. 1999b. Structure-function of the ORF 7 protein of porcine reproductive and respiratory syndrome virus in the viral capsid assembly, pp. 37-38. **Proceeding of the International Symposium on PRRS and Aujeszky's Disease**, Ploufragan, France.

Xue, Q., Y.G. Zhao, Y.J. Zhou, H.J. Qiu, Y.F. Wang, D.L. Wu, Z.J. Tian and G.Z. Tong. 2004. Immune responses of swine following DNA immunization with plasmids encoding porcine reproductive and respiratory syndrome virus ORFs 5 and 7 and porcine IL-2 and IFNγ. **Vet. Immun. Immunopath.** 102: 291-298.

Yaeger, M.J., T. Prieve, J. Collins, J. Christopher-Hennings, E. Nelson and D. Benfield. 1993. Evidence for the transmission of porcine reproductive and respiratory syndrome (PRRS) virus in boar semen. **Swine Health Prod**. 1: 7-9.

Yoon, I.J., H.S. Joo, W.T. Christianson, K.S. Kim, J.E. Collins and R.B. Morrison. 1992. An indirect fluorescent antibody test for the detection of antibody to swine infertility and respiratory syndrome virus in swine sera. **J. Vet. Diagn. Invest.** 4: 144-147.

Yoon, K.J., H.S. Joo, S.M. Goyal, and T.W. Molitor. 1994. A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in swine sera. **J. Vet. Diagn. Invest.** 6: 289-292.

Yoon, K.J., L.L. Wu, J.J. Zimmerman, H.T. Hill and K.B. Platt. 1996. Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in oigs. **Viral Immunol.** 9: 51-63.

Zhou, Y.J., G.Z. Tong, Q. Xue, H.J. Qiu, Y.F. Wang and Y.G. Zhao. 2002. High-level expression and primary application of nucleocapsid gene of PRRSV CH-1a strain in prokaryotic expression system. **Chin. J. Prev. Vet. Med.** 24: 401-404.

Zimmerman, J., W. Epplerson, R.W. Wills and J.D. McKean. 1997. Results of the recent survey of the membership of the AASP for ortbreaks of sow abortion and mortality. **Swine Health Prod..** 5: 74-75.

APPENDICES

Appendix A

Number of serum samples and raw data of ELISA

Appendix Table A1 Sorces of the serum samples and amount sera for each method

Samples	RT-nPCR (300 samples)	ELISA test for vilidation (288 samples)	ELISA test for HerdChek comparisom (300 samples)
North	50	25	40
South	40	25	40
East	70	88	80
West	80	100	80
North East	60	50	60

Appendix Table A2 Raw data for evaluating rN-I ELISA reactivity

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
SR8	0.022988506	0.09	0.078	0.95	0.95	-0.335195531
TV3	0.058516196	0.097	0.1	0.95	0.055	-0.111731844
MN5	0.031347962	0.07	0.076	0.95	0.055	-0.61452514
MN10	0.089864159	0.14	0.15	0.95	0.055	-0.558659218
142	-0.054600607	0.154	0.163	0.87	0.048	-2.243589744
TV5	0.087774295	0.048	0.05	0.87	0.048	-0.384615385
WL4	-0.023174971	0.061	0.067	0.87	0.048	-1.923076923
MN7	-0.489028213	0.051	0.066	0.87	0.048	-3.846153846
PJ10	0.085747393	0.046	0.047	0.87	0.048	-0.128205128
52	-0.071055381	0.048	0.049	0.87	0.048	-1.41025641
TV6	0.048066876	0.049	0.05	0.87	0.048	-0.384615385
TV7	0.014629049	0.051	0.052	0.87	0.048	-0.256410256
PJ8	0.053302433	0.052	0.062	0.87	0.048	-1.666666667
PJ2	0.044032445	0.048	0.05	0.87	0.048	-0.769230769
PV3	0.299292214	0.139	0.11	0.95	0.055	3.631284916
SS2	0.21107628	0.178	0.11	0.95	0.055	7.318435754
SS3	0.248693835	0.16	0.129	0.95	0.055	4.189944134
SS4	0.317659352	0.125	0.094	0.95	0.055	3.519553073
SS6	0.326018809	0.133	0.99	0.95	0.055	3.575418994
SS7	0.374085684	0.135	0.12	0.95	0.055	1.173184358
BM1	0.229885057	0.144	0.129	0.95	0.055	1.452513966
BM3	0.10031348	0.1	0.087	0.95	0.055	2.458100559
BM5	0.179728318	0.131	0.094	0.95	0.055	3.854748603
BM7	0.234064786	0.153	0.125	0.95	0.055	3.072625698
TV1	0.225705329	0.16	0.12	0.95	0.055	3.687150838
SS8	0.158829676	0.153	0.125	0.95	0.055	3.072625698
SS10	0.169278997	0.188	0.11	0.95	0.055	4.357541899
SR4	0.227795193	0.18	0.14	0.95	0.055	0.391061453
SR5	0.117032393	0.159	0.12	0.95	0.055	6.536312849
SR6	0.183908046	0.11	0.072	0.95	0.055	1.675977654
TV4	0.217345873	0.099	0.62	0.95	0.055	3.296089385

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
TV8	0.328108673	0.106	0.102	0.95	0.055	0.446927374
TV9	0.244514107	0.19	0.145	0.95	0.055	4.301675978
TV10	0.127481714	0.126	0.095	0.95	0.055	4.022346369
MN1	0.234064786	0.2	0.175	0.95	0.055	2.793296089
PN1	0.164542294	0.182	0.173	0.95	0.055	1.414141414
PN3	0.259559676	0.12	0.104	0.95	0.055	3.434343434
PN4	0.222479722	0.132	0.096	0.95	0.055	7.373737374
PN8	0.048667439	0.148	0.111	0.95	0.055	7.171717172
MN6	0.121212121	0.199	0.16	0.95	0.055	4.949494949
PJ1	0.162224797	0.11	0.104	0.95	0.055	1.01010101
MN8	0.355276907	0.096	0.066	0.95	0.055	5.656565657
PJ5	0.136732329	0.109	0.089	0.95	0.055	7.373737374
WL1	0.034762457	0.095	0.08	0.95	0.055	3.535353535
WL3	0.111239861	0.1	0.076	0.95	0.055	4.646464646
WL6	0.106604867	0.178	0.15	0.95	0.055	5.353535354
WL8	0.250289687	0.116	0.092	0.95	0.055	4.141414141
WL10	0.201622248	0.114	0.088	0.95	0.055	5.656565657
ST4	0.162224797	0.105	0.077	0.95	0.055	4.949494949
ST5	0.10428737	0.117	0.08	0.95	0.055	5.050505051
ST6	0.053302433	0.11	0.08	0.95	0.055	6.666666667
ST8	0.090382387	0.101	0.08	0.95	0.055	5.151515152
ST9	0.099652375	0.176	0.147	0.95	0.055	4.747474747
4อท	0.393974508	0.12	0.08	0.95	0.055	4.646464646
4ค	0.342989571	0.108	0.08	0.95	0.055	4.949494949
5ค	0.222479722	0.15	0.123	0.95	0.055	7.575757576
6ค	0.048667439	0.2	0.172	0.95	0.055	4.04040404
7ค	0.121212121	0.126	0.099	0.95	0.055	7.575757576
8ค	0.162224797	0.16	0.145	0.95	0.055	7.070707071
5อท	0.355276907	0.14	0.108	0.95	0.055	5.95959596
9ค	0.136732329	0.157	0.123	0.95	0.055	6.767676768

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
138	0.143579373	0.132	0.1	0.95	0.055	1.717171717
139	0.22851365	0.132	0.101	0.95	0.055	5.656565657
6อท	0.106604867	0.084	0.078	0.95	0.055	4.04040404
7อท	0.250289687	0.177	0.117	0.95	0.055	1.818181818
8อท	0.201622248	0.183	0.184	0.95	0.055	5.757575758
9อท	0.106604867	0.191	0.162	0.95	0.055	7.373737374
SS5	0.482758621	0.209	0.12	0.95	0.055	15.35353535
BM10	0.399164054	0.209	0.095	0.95	0.055	17.47474747
PJ7	0.366164542	0.209	0.12	0.95	0.055	15.35353535
10อท	0.222479722	0.218	0.12	0.95	0.055	18.08080808
MN4	0.570532915	0.23	0.093	0.95	0.055	29.09090909
141	0.602628918	0.234	0.095	0.95	0.055	25.85858586
146	0.825075834	0.2	0.09	0.95	0.055	20.50505051
156	0.59049545	0.211	0.09	0.95	0.055	25.15151515
PN9	0.604866744	0.231	0.086	0.87	0.048	28.48484848
172	0.622851365	0.211	0.078	0.87	0.048	27.67676768
BS1	0.498886414	0.231	0.108	0.87	0.048	24.64646465
BM2	0.566353187	0.264	0.117	0.87	0.048	28.78787879
SR7	0.587251829	0.247	0.11	0.87	0.048	26.76767677
MN2	0.693834901	0.228	0.12	0.87	0.048	24.04040404
SR9	0.597701149	0.247	0.11	0.87	0.048	25.95959596
BM8	0.76907001	0.247	0.09	0.87	0.048	27.37373737
BM9	0.541274817	0.206	0.1	0.95	0.055	21.51515152
AR1	0.6875	0.252	0.09	0.95	0.055	32.32323232
AR2	0.701388889	0.283	0.09	0.95	0.055	37.57575758
AR3	0.761290323	0.257	0.083	0.87	0.048	34.34343434
AR4	0.761290323	0.223	0.08	0.87	0.048	31.31313131
AR5	0.7	0.254	0.091	0.87	0.048	35.85858586
AR6	0.726057906	0.313	0.125	0.87	0.048	36.86868687
AR7	0.76169265	0.313	0.125	0.87	0.048	37.37373737

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
AR8	0.608294931	0.313	0.114	0.87	0.048	39.09090909
AR9	0.64516129	0.313	0.114	0.95	0.055	38.38383838
WL7	0.614136732	0.259	0.1	0.95	0.055	32.42424242
43	0.547884187	0.313	0.124	0.95	0.055	37.77777778
45	0.556792873	0.313	0.143	0.95	0.055	33.73737374
112	0.690104167	0.341	0.12	0.95	0.055	42.02020202
113	0.7265625	0.319	0.08	0.95	0.055	47.97979798
114	0.692708333	0.319	0.114	0.95	0.055	41.91919192
115	0.723958333	0.34	0.111	0.95	0.055	45.65656566
116	0.7265625	0.321	0.111	0.95	0.055	42.92929293
117	0.619791667	0.339	0.111	0.95	0.055	45.25252525
118	0.786458333	0.37	0.111	0.95	0.055	45.75757576
119	0.721354167	0.323	0.08	0.95	0.055	49.19191919
120	0.744791667	0.317	0.11	0.95	0.055	41.61616162
121	0.729166667	0.309	0.1	0.95	0.055	42.32323232
122	0.611979167	0.317	0.08	0.95	0.055	48.68686869
123	0.6328125	0.32	0.08	0.95	0.055	49.19191919
124	0.786458333	0.378	0.111	0.95	0.055	45.55555556
125	0.65625	0.321	0.12	0.95	0.055	42.02020202
126	0.631578947	0.33	0.08	0.95	0.055	49.09090909
127	0.631578947	0.337	0.11	0.95	0.055	42.92929293
128	0.631578947	0.309	0.1	0.95	0.055	42.32323232
129	0.736842105	0.323	0.08	0.95	0.055	47.27272727
130	0.606060606	0.323	0.111	0.95	0.055	42.52525253
131	0.787878788	0.323	0.12	0.95	0.055	41.01010101
132	0.606060606	0.314	0.08	0.95	0.055	47.77777778
133	0.877505568	0.38	0.1	0.87	0.048	58.28282828
135	0.894009217	0.4	0.116	0.87	0.048	51.41414141
136	0.829493088	0.442	0.157	0.87	0.048	58.08080808
137	0.919270833	0.38	0.14	0.87	0.048	53.23232323
138	0.90625	0.38	0.08	0.87	0.048	58.88888889

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
139	0.833333333	0.352	0.09	0.87	0.048	54.24242424
140	0.958333333	0.379	0.101	0.87	0.048	53.53535354
141	0.848958333	0.38	0.1	0.87	0.048	58.28282828
143	0.934210526	0.4	0.116	0.87	0.048	51.41414141
144	0.960526316	0.442	0.157	0.87	0.048	58.08080808
145	0.98245614	0.38	0.14	0.87	0.048	53.23232323
146	0.973684211	0.38	0.08	0.95	0.055	58.88888889
147	0.929824561	0.352	0.09	0.95	0.055	54.24242424
148	0.925438596	0.379	0.101	0.95	0.055	53.53535354
149	0.912280702	0.352	0.09	0.95	0.055	54.24242424
150	0.877192982	0.4	0.08	0.95	0.055	59.8989899
151	0.98245614	0.379	0.09	0.95	0.055	55.15151515
152	0.99122807	0.38	0.101	0.95	0.055	58.08080808
SS1	0.867293626	0.433	0.09	0.87	0.048	66.36363636
143	0.841253792	0.467	0.118	0.87	0.048	67.27272727
PJ3	0.769409038	0.455	0.12	0.87	0.048	66.66666667
PJ4	0.799536501	0.44	0.08	0.87	0.048	68.98989899
PN7	0.720741599	0.455	0.09	0.87	0.048	69.49494949
PN2	0.739281576	0.442	0.101	0.87	0.048	69.09090909
166	0.897876643	0.442	0.09	0.87	0.048	69.49494949
173	0.879676441	0.424	0.09	0.87	0.048	65.65656566
ST10	0.984936269	0.437	0.101	0.87	0.048	67.37373737
1อท	0.850521437	0.44	0.09	0.87	0.048	68.68686869
PJ9	0.998841251	0.442	0.116	0.87	0.048	65.95959596
2ค	1.077636153	0.442	0.157	0.87	0.048	63.13131313
57	0.895833333	0.442	0.14	0.87	0.048	61.61616162
68	0.95483871	0.455	0.116	0.87	0.048	66.96969697
69	0.8	0.442	0.116	0.87	0.048	65.95959596
70	0.914572864	0.442	0.116	0.87	0.048	65.95959596
AA1	0.874371859	0.424	0.08	0.87	0.048	69.49494949

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
AA2	0.819599109	0.437	0.09	0.87	0.048	68.98989899
AA3	0.801781737	0.44	0.09	0.87	0.048	68.68686869
AA4	0.855233853	0.433	0.09	0.87	0.048	64.74747475
AA5	0.846325167	0.46	0.116	0.87	0.048	69.6969697
AA6	0.881959911	0.455	0.116	0.87	0.048	66.96969697
ST7	1.73580533	0.446	0.08	0.95	0.055	72.72727273
8ค	1.427578216	0.51	0.132	0.87	0.048	76.66666667
WL2	1.181923523	0.49	0.117	0.87	0.048	78.48484848
ST2	1.154113557	0.52	0.116	0.87	0.048	79.6969697
PJ6	1.028968714	0.498	0.157	0.87	0.048	74.34343434
PN6	1.429895713	0.52	0.14	0.87	0.048	78.08080808
SR2	1.375130617	0.505	0.14	0.87	0.048	72.52525253
153	1.403437816	0.507	0.132	0.87	0.048	78.58585859
140	1.104145602	0.514	0.117	0.87	0.048	78.58585859
133	1.205257836	0.52	0.129	0.87	0.048	78.98989899
134	1.795753286	0.505	0.157	0.87	0.048	74.64646465
136	1.154701719	0.507	0.14	0.87	0.048	76.06060606
144	1.336703741	0.514	0.14	0.87	0.048	73.63636364
145	1.714863498	0.51	0.132	0.87	0.048	76.66666667
147	1.274014156	0.49	0.117	0.87	0.048	78.48484848
148	1.371081901	0.52	0.129	0.87	0.048	76.16161616
149	1.662285137	0.498	0.157	0.87	0.048	74.34343434
150	1.243680485	0.52	0.14	0.87	0.048	78.08080808
152	1.858442872	0.505	0.14	0.87	0.048	72.52525253
162	1.389282103	0.507	0.157	0.87	0.048	78.18181818
157	1.530839232	0.514	0.14	0.87	0.048	73.63636364
151	2.022244692	0.52	0.14	0.87	0.048	78.08080808
137	1.93124368	0.505	0.132	0.87	0.048	75.05050505
135	2.121334681	0.507	0.117	0.87	0.048	78.98989899
154	2.275025278	0.514	0.129	0.87	0.048	74.54545455

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
155	2.29322548	0.544	0.13	0.87	0.048	84.14141414
174	1.130434783	0.559	0.17	0.87	0.048	83.23232323
10ค	1.799797776	0.615	0.21	0.87	0.048	81.61616162
158	2.475227503	0.575	0.13	0.87	0.048	89.8989899
159	2.394337715	0.6	0.14	0.87	0.048	86.36363636
160	2.056622851	0.544	0.13	0.87	0.048	84.14141414
161	2.042467139	0.589	0.14	0.87	0.048	82.22222222
163	2.468041237	0.615	0.17	0.87	0.048	85.65656566
164	1.80586451	0.589	0.14	0.87	0.048	83.33333333
165	2.432760364	0.587	0.14	0.87	0.048	81.81818182
167	2.101112235	0.577	0.13	0.87	0.048	89.8989899
168	1.189079879	0.531	0.13	0.87	0.048	83.43434343
169	1.686552073	0.544	0.13	0.87	0.048	84.14141414
170	1.70677452	0.559	0.17	0.87	0.048	83.23232323
171	1.557128413	0.615	0.21	0.87	0.048	81.61616162
SS9	2.175548589	0.575	0.13	0.87	0.048	89.8989899
SR10	2.033437827	0.6	0.14	0.87	0.048	86.36363636
WL5	2.289687138	0.544	0.13	0.87	0.048	84.14141414
1ค	2.774044032	0.589	0.14	0.87	0.048	82.22222222
2อท	2.025492468	0.615	0.17	0.87	0.048	85.65656566
3อท	2.611819235	0.589	0.14	0.87	0.048	83.33333333
71	2.5625	0.587	0.14	0.87	0.048	81.81818182
72	2.215277778	0.577	0.13	0.87	0.048	89.8989899
73	3.930555556	0.531	0.13	0.87	0.048	83.43434343
74	2.173611111	0.575	0.13	0.95	0.055	89.6969697
75	2.395833333	0.531	0.13	0.95	0.055	83.43434343
76	2.979166667	0.544	0.13	0.95	0.055	84.14141414
77	2.944444444	0.559	0.13	0.95	0.055	87.67676768
78	2.069444444	0.587	0.13	0.95	0.055	89.5959596
79	3.520833333	0.575	0.14	0.95	0.055	80.4040404

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
80	3.069444444	0.6	0.17	0.95	0.055	82.32323232
81	1.055555556	0.589	0.14	0.95	0.055	83.33333333
82	1.347222222	0.587	0.19	0.95	0.055	86.86868687
83	1.618055556	0.66	0.0.12	0.95	0.055	96.06060606
84	1.076388889	0.6	0.12	0.95	0.055	95.05050505
85	2.8125	0.615	0.13	0.95	0.055	99.19191919
86	1.520833333	0.599	0.17	0.95	0.055	90.70707071
87	1.006944444	0.667	0.213	0.95	0.055	91.11111111
88	2.638888889	0.689	0.25	0.95	0.055	91.41414141
89	2.0625	0.589	0.11	0.95	0.055	96.06060606
90	1.819444444	0.6	0.13	0.95	0.055	95.05050505
91	2.652777778	0.615	0.13	0.95	0.055	99.19191919
92	1.409722222	0.599	0.13	0.95	0.055	96.76767677
93	1.430555556	0.667	0.213	0.95	0.055	91.11111111
94	1.680555556	0.689	0.25	0.95	0.055	91.41414141
95	2.631944444	0.599	0.17	0.95	0.055	90.70707071
96	2.694444444	0.667	0.213	0.95	0.055	91.11111111
97	2.868055556	0.689	0.25	0.95	0.055	91.41414141
98	1.256944444	0.589	0.11	0.95	0.055	96.06060606
99	1.083333333	0.6	0.13	0.95	0.055	95.05050505
100	2.472222222	0.615	0.13	0.95	0.055	99.19191919
101	1.909722222	0.599	0.13	0.87	0.048	96.76767677
102	1.555555556	0.589	0.11	0.95	0.055	96.06060606
103	2.326388889	0.6	0.13	0.95	0.055	95.05050505
104	2.583333333	0.615	0.13	0.95	0.055	99.19191919
105	2.025806452	0.599	0.13	0.95	0.055	96.76767677
106	3.793548387	0.667	0.213	0.95	0.055	91.11111111
107	3.432258065	0.689	0.25	0.95	0.055	91.41414141
108	1.432258065	0.599	0.1	0.95	0.055	96.76767677
109	1.096774194	0.667	0.213	0.95	0.055	91.11111111

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
110	2.025806452	0.689	0.25	0.95	0.055	91.41414141
111	2.55483871	0.589	0.11	0.95	0.055	96.06060606
445	1.987096774	0.6	0.13	0.95	0.055	95.05050505
446	2.051612903	0.615	0.13	0.95	0.055	99.19191919
447	2.64516129	0.599	0.13	0.87	0.048	96.76767677
448	3.148387097	0.589	0.11	0.95	0.055	96.06060606
449	5.070967742	0.6	0.13	0.95	0.055	95.05050505
223	4.851612903	0.91	0.061	0.95	0.055	179.5959596
224	2.309677419	0.84	0.061	0.95	0.055	173.8383838
225	7.290322581	0.95	0.07	0.95	0.055	171.2121212
226	2.529032258	1.09	0.05	0.95	0.055	179.0909091
227	7.303225806	1.18	0.086	0.95	0.055	231.7171717
228	2.374193548	1.21	0.112	0.95	0.055	218.989899
229	4.516129032	0.98	0.731	0.95	0.055	105.7575758
230	3.419354839	0.93	0.112	0.87	0.048	173.4343434
231	2.580645161	0.95	0.07	0.87	0.048	171.2121212
232	6.374193548	1.09	0.05	0.87	0.048	179.0909091
233	1.367741935	1.18	0.086	0.87	0.048	231.7171717
234	3.741935484	1.21	0.112	0.87	0.048	218.989899
235	6.193548387	0.98	0.731	0.87	0.048	105.7575758
236	6.077419355	0.93	0.112	0.87	0.048	173.4343434
237	1.638709677	1.18	0.086	0.87	0.048	231.7171717
238	1.432258065	1.21	0.112	0.87	0.048	218.989899
239	2.709677419	0.98	0.731	0.87	0.048	105.7575758
240	5.393548387	0.93	0.112	0.87	0.048	173.4343434
241	4.967741935	0.95	0.07	0.87	0.048	171.2121212
242	4.361290323	1.09	0.05	0.87	0.048	179.0909091
243	4.825806452	1.18	0.086	0.87	0.048	231.7171717
244	2.270967742	1.21	0.112	0.87	0.048	218.989899
245	4.206451613	0.98	0.731	0.87	0.048	105.7575758
246	2.5125	0.93	0.112	0.95	0.055	173.4343434

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
247	2.55	1.29	0.17	0.95	0.055	224.7474747
248	7.0125	1	0.089	0.95	0.055	183.7171717
249	4.8	0.98	0.731	0.95	0.055	105.7575758
250	7.8125	0.93	0.112	0.95	0.055	173.4343434
251	7	0.95	0.07	0.95	0.055	171.2121212
252	1.2625	1.09	0.05	0.95	0.055	179.0909091
253	2.2375	1.18	0.086	0.87	0.048	231.7171717
254	3.1	1.18	0.086	0.87	0.048	231.7171717
255	4.15	1.21	0.112	0.87	0.048	218.989899
256	7.1125	0.98	0.731	0.87	0.048	105.7575758
257	2.3	0.93	0.112	0.87	0.048	173.4343434
258	1.2125	1.29	0.17	0.87	0.048	224.7474747
259	1.6625	1	0.089	0.87	0.048	183.7171717
260	1.0125	0.98	0.731	0.87	0.048	105.7575758
261	1.0625	0.93	0.112	0.87	0.048	173.4343434
262	3.3375	0.95	0.07	0.87	0.048	171.2121212
263	4.0625	1.09	0.05	0.87	0.048	179.0909091
264	5.658291457	1.18	0.086	0.87	0.048	231.7171717
247	2.55	1.29	0.17	0.95	0.055	224.7474747
248	7.0125	1	0.089	0.95	0.055	183.7171717
249	4.8	0.98	0.731	0.95	0.055	105.7575758
250	7.8125	0.93	0.112	0.95	0.055	173.4343434
251	7	0.95	0.07	0.95	0.055	171.2121212
252	1.2625	1.09	0.05	0.95	0.055	179.0909091
253	2.2375	1.18	0.086	0.87	0.048	231.7171717
254	3.1	1.18	0.086	0.87	0.048	231.7171717
255	4.15	1.21	0.112	0.87	0.048	218.989899
256	7.1125	0.98	0.731	0.87	0.048	105.7575758
257	2.3	0.93	0.112	0.87	0.048	173.4343434
258	1.2125	1.29	0.17	0.87	0.048	224.7474747
259	1.6625	1	0.089	0.87	0.048	183.7171717

Appendix Table A2 (Continued)

Sample name	IDEXX S/P ratio	OD PRRS (average)	OD Untranform *E.coli* (average)	OD positive (average)	OD negative (average)	%Reactivity
260	1.0125	0.98	0.731	0.87	0.048	105.7575758
261	1.0625	0.93	0.112	0.87	0.048	173.4343434
262	3.3375	0.95	0.07	0.87	0.048	171.2121212
263	4.0625	1.09	0.05	0.87	0.048	179.0909091
264	5.658291457	1.18	0.086	0.87	0.048	231.7171717

Appendix B

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

- pH 8.3 and bring to 1 litre
with distilled water.

- 10X loading buffer / dye 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-$3H_20$
  0.04 M Na_2EDTA; 14.9 g

Adjust pH with glacial acetic acid to
  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. Buffers for Protein Purification

Lysis buffer B (1 litre):

100 mM NaH2PO4;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. SDS-PAGE Reagents

- 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

- Diaminobenzidine (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 C

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°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°c for 5 min, then on ice for 10 min.
6. Add 1 ml of LB and incubate the tube at 37°c for 1 hour.
7. Plate the cells on the appropriate selective or differential media.

2. Transformation (Sambrook *et al.*, 1989)

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 µg/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 al.*, 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 µl
   - vector DNA : insert DNA (1:3)
   - autoclaved distilled water to 20 µl
2. Add 1.0 µl (0.1 unit) of T4 DNA ligase (Promega, USA.). 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. Rapid Alkaline E xtraction Method for Plasmid Isolation (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 20 µl 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. Restriction Endonuclease Assay (Fermentas, USA)

This procedure is used for cutting the target DNA.

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 *Eco*RI 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 (20 ng/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. SDS-Polyacrylamide Get Electrophoresis (SDS-PAGE) (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 µl
TEMED	5.3 µl

- Stacking gel

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 µl
TEMED	5 µl

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 – 25 µl (1 – 10 µg 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)

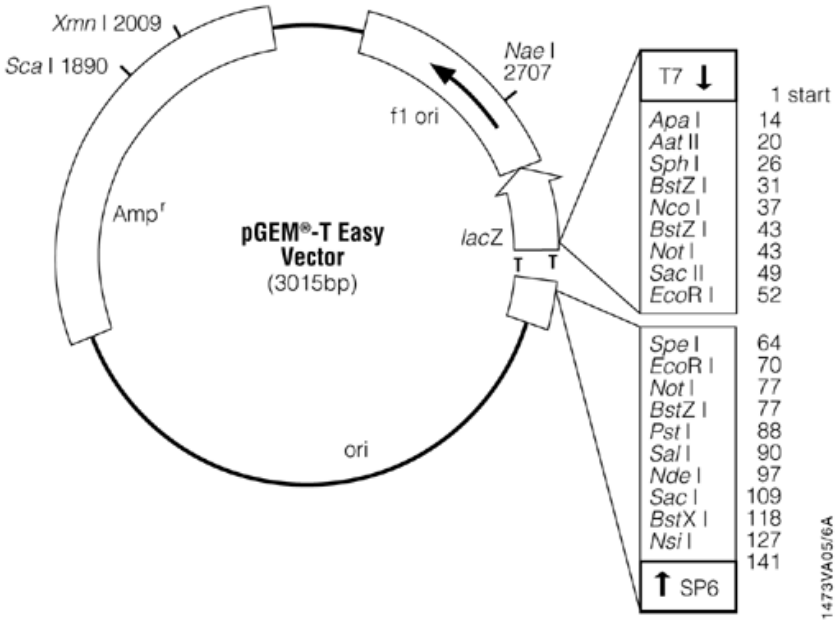
9. Western Transfer and Blotting (Cochet *et al.*, 1998)

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 (25 mM Tris base, 190 mM 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.

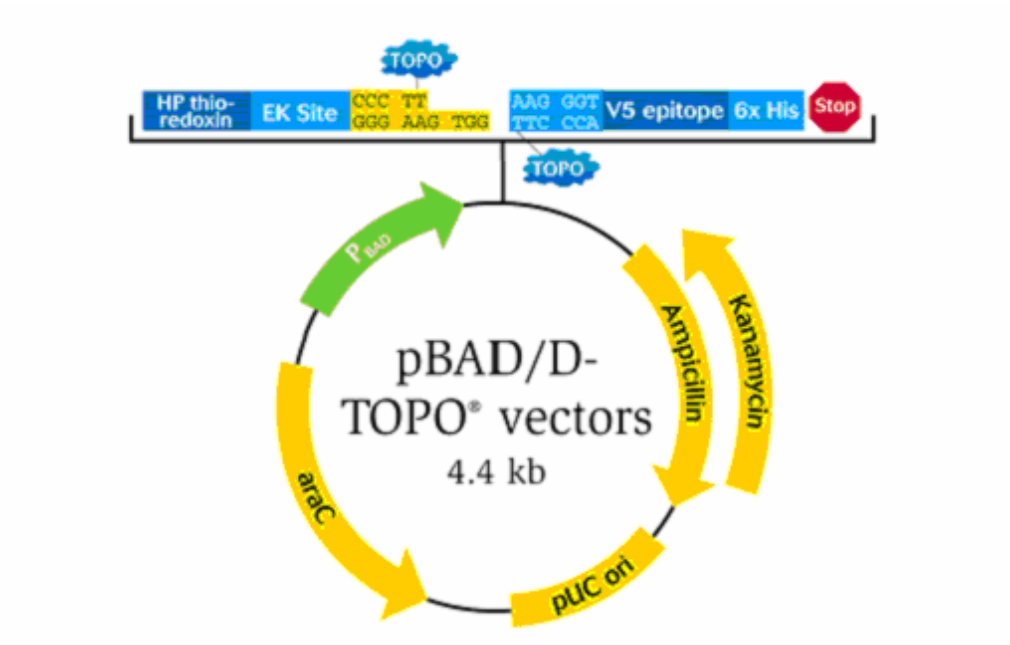
7. Dilute the secondary antibody (rabbit anti-goat IgG (1:1,000) or goat anti-mouse IgG (1:500) conjugated with peroxidase). Decant the primary antibody from the blot, add the secondary antibody solution, and incubate with agitation for one hour at room temperature (45 minutes at 37°C)
8. Decant the 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.

Appendix D

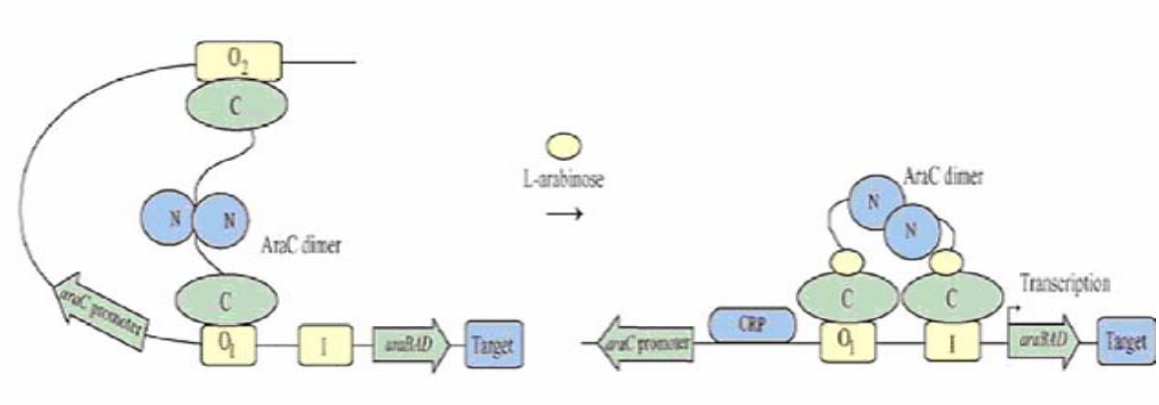
Cloning and Expression vector



Appendix Figure D1 The pGEM®-T Easy cloning vector (Promega, USA)



Appendix Figure D2 The pBAD Directional TOPO® expression vectors (Invitrogen, USA)



Appendix Figure D3 Regulation of the arabinose operon in *E. coli***.** The *ara*BAD promotor used in the pBAD/D-TOPO® vectors is both positively and negatively regulated by the product of the *ara*C gene (Schleif, 1992). AraC is a transcriptional regulator that forms a complex with L-arabinose. In the absence of arabinose the AraC dimer contacts the O_2 and I_2 half sites of the *ara*BAD operon, forming a 210 bp DNA loop. Arabinose binds to AraC and causes the protein to release the O_2 site and bind the I_2 site which is adjacent to the I_1 site. This releases the DNA loop and allows transcription to begin. The cAMP activator protein (CAP) –cAMP complex binds to the DNA and stimulates binding of AraC to I_1 and I_2.

CURRICULUM VITAE

NAME : **Mr. Picharn Theveethivarak**

BIRTH DATE : **November 21, 1980**

BIRTH PLACE : **Bangkok, Thailand**

EDUCATION : **YEAR** **INSTITUTION** **DEGREE/DIPLOMA**

: **2004** **Chulalongkorn Univ.** **D.V.M.**

POSITION/TITLE : **-**

WORK PLACE : **-**

SCHOLARSHIP/AWARDS: **-**