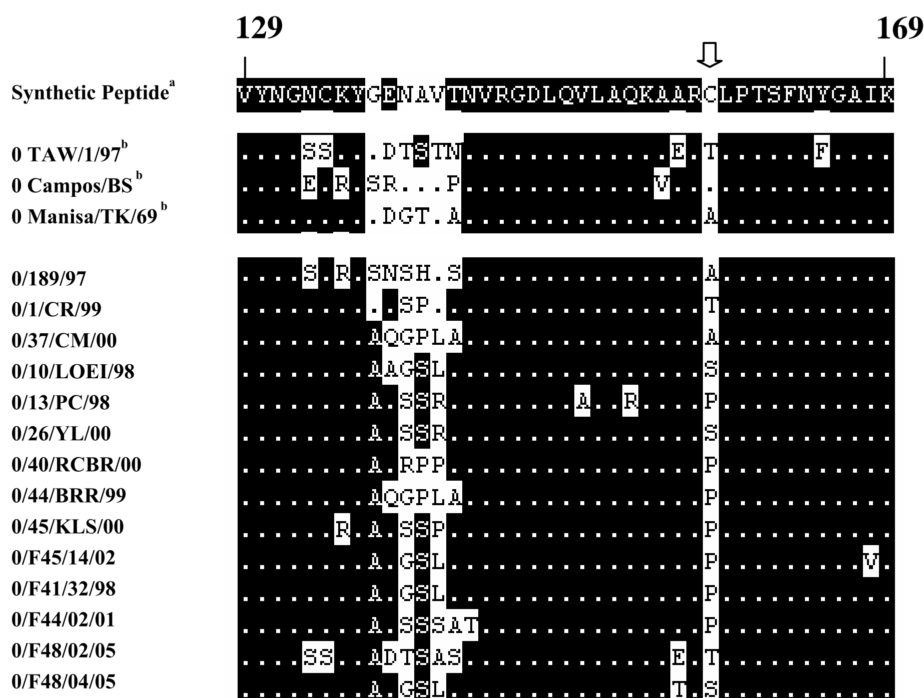

Figure 19 Comparison of amino acid residues among synthetic peptide vaccine, O/189 (vaccine strain) and amino acid sequences of viruses isolated in Thailand during 1998 to 2005. The position 158 (arrow) of peptide was modified to cysteine. Dots showed the identical amino acid sequence with peptide vaccine. The black marks were represented as the conserved sequences.

A = amino acid sequence of synthetic peptide vaccine (Wang *et al*., 2002).

B = amino acid sequence of viruses was evaluated protection from peptide vaccine (Wang *et al*., 2002).

3. 3AB Enzymed Linked Immunosorbent Assay

The nonstructural 3AB recombinant protein was partially purified by urea gradients, dissolved in SDS solution and used to coat the ELISA plates (KU-ELISA 3AB test kit). The antigens were used to validate with the sera from both infected and non-infected bovine and swine using the indirect ELISA (iELISA) technique. All reagents were ascertained by prior checkerboard titration. A positive control serum was derived from the pool positive sera collected from natural infected bovine and swine.

3.1 Evaluation of Assay Repeatability

Each six negative and positive bovine sera were analyzed for 4 replicates to determine the repeatability of intra-assay (within plate) and inter-assay (between run) of KU-ELISA 3AB test kit (Table 11). The results of both assays showed repeatable. The repeatability of negative bovine sera was indicated by low variability among each replicate of each sample. The repeatability of positive bovine sera expressed as coefficients of variant (CV) was ranged from 4.3 to 8.4% for intra-assay and 2 to11% for inter-assay.

3.2 Evaluation of Dry Coating

The KU-ELISA3AB test kit bound was blocked with blocking buffer and completely dried before using. Either three of swine or bovine sera were evaluated for the antigen stability during 2 month interval at dark and cool condition (4°C) after antigen coating by eight replications of each samples. The results showed low variation of S/P ratio of all sera which the CVs of four positive sera were ranged from 4.78 to 12.74 % (Table 12).

Table 11 showed the repeatability of KU- ELISA 3AB test kit for intra-assay and inter-assay for four replications using negative and positive bovine sera. The results showed as the mean of S/P ratio ± S.D.

Samples	Mean S/P ratio ± S.D.	
	Intra assay	Inter assay
Negative sera	0.036 ±0.007	0.033 ±0.003
	0.010 ±0.001	0.008 ±0.005
	0.038 ±0.025	0.028 ±0.007
	0.062 ±0.022	0.054 ±0.007
	0.013 ±0.021	0.008 ±0.005
	0.022 ±0.023	0.020 ±0.005
Positive sera	2.932 ±0.127	3.004 ±0.173
	1.409 ±0.082	1.480 ±0.143
	0.606 ±0.038	0.640 ±0.071
	2.094 ±0.107	2.178 ±0.151
	3.611 ±0.302	3.664 ±0.074
	0.951 ±0.061	0.995 ±0.085

Table 12 showed the stability results of KU- ELISA 3AB test kit during 2 month interval. The results showed as the mean of S/P ratio ± S.D. and coefficients of variant (CV).

Number	Time of antigen dry coating (mean ± S.D.)			Mean ± S.D. of all times	Coefficients of variant (CV)
	First day	1st month	2nd month		
1[a]	0.98 ± 0.07	0.91 ± 0.04	1.16 ± 0.10	1.02 ± 0.13	12.75
2[a]	1.07 ± 0.10	0.91 ± 0.05	1.07 ± 0.13	1.02 ± 0.09	8.82
3[b]	0	0 ± 0.02	0.03 ± 0.04	0.01 ± 0.09	NA
4[c]	2.40 ± 0.23	2.54 ± 0.21	2.58 ± 0.28	2.51 ± 0.12	4.78
5[c]	2.42 ± 0.28	2.50 ± 0.20	2.66 ± 0.25	2.53 ± 0.02	0.79
6[d]	0.18 ± 0.07	0.06 ± 0.02	0.17 ± 0.05	0.14 ± 0.07	NA

a = positive swine

b = negative swine

c = positive bovine

d = negative bovine

NA = No analysis

3.3 Establishing S/P Ratio for Swine Sera

The one hundred and ninety five sera of imported swine without history of FMD infection were used to evaluate cut off value of the S/P ratio (Figure 20). This group of swine had the mean of 0.05 mean, the S.D. of 0.05, the minimum value of -0.07 and the maximum value of 0.23. The majority of swine (193/195) had the S/P ratio less than 2.0 (mean+3SD) which gave the specificity of 98.97%. Two swine out of one hundred and ninety five swine gave the S/P value 0.22 and 0.23. According to this result, the 100% of specificity can be obtained by using the cut off value at 4 S.D of the mean (0.25) (Table 13). One hundred and forty four sera of vaccinated sows from the farms without the history of FMD outbreak for more than five years were used to evaluate. These sows had vaccinated for at least 10 vaccinations. The S/P rations of these sows were exhibited a normal distribution similar to imported swine sera (Figure 21). The majority of sera had the S/P ratio less than 0.2 except one sample that had the S/P ratio of 0.39. The specificity of vaccinated sows was 99.30 %(Table 13). These sera gave the mean of S/P ratio ± 1S.D. of 0.013 ± 0.057.

The one hundred and two swine sera collected three weeks after FMD outbreak in a farm were used to evaluate the antibody response to 3AB recombinant protein using KU- ELISA 3AB test kit. The frequency distribution of S/P ratio was shown in figure 21. The mean of S/P ratio (±S.D.) of infected swine was 1.50 ± 1.00. There were 11 and 13 samples negative samples when the cut off value was set as of mean +3S.D. and mean +4S.D., respectively. Ten samples out of the 11 negative samples using cut off value at mean +3S.D. gave the negative by using UBI® FMDV NS NIA.

The cut off value to give the satisfied specificity and sensitivity was 0.2 (mean +3S.D.) for KU-ELISA 3AB test kit as it gave the high percentage both of specificity (98.97-99.30%) for imported swine and vaccinated sows and specificity (90.09%) for natural infected swine (Table 13).. This cut off value was used for other experiment.

Seventy four infected swine sera were evaluated for the agreement among KU-ELISA 3AB test kit and two commercial test kits (ELISA-A and ELISA-B) (Table 14). In the agreement test, the cut off value of 30% was used to kappa calculation for ELISA-A test kit which had higher correlative value among other tests. The results showed kappa values of 0.479 between KU-ELISA 3AB and ELISA-A, 0.835 between KU-ELISA 3AB and ELISA-B and 0.367 between ELISA-A and ELISA-B. ELISA-A kit test had the lowest correlative values for both KU-ELISA 3AB and ELISA-B kit tests. However, the correlative value between KU-ELISA 3AB and ELISA-B kit tests was in perfect agreement (Table 14).

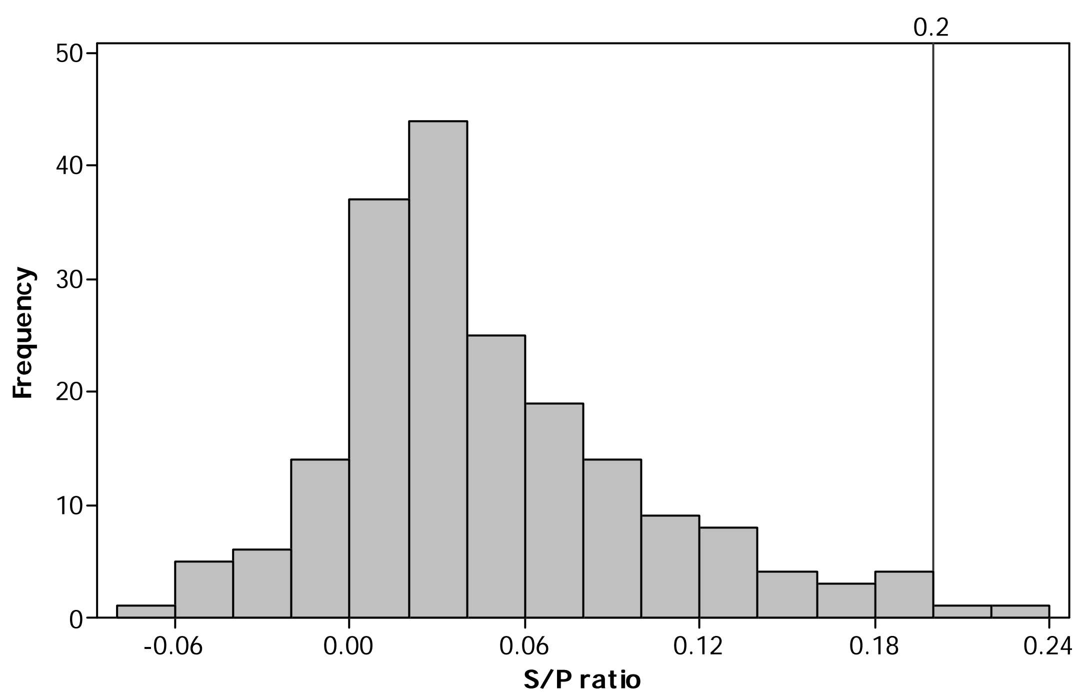

Figure 20 Diagram of antibody response in non-infected sera from imported swine using KU-ELISA3AB kit test. The result was expressed as the S/P ratio. The cut off value was indicated as the vertical line. (n = 195, mean = 0.05, S.D. = 0.05, minimum value = -0.07 and maximum value = 0.23)

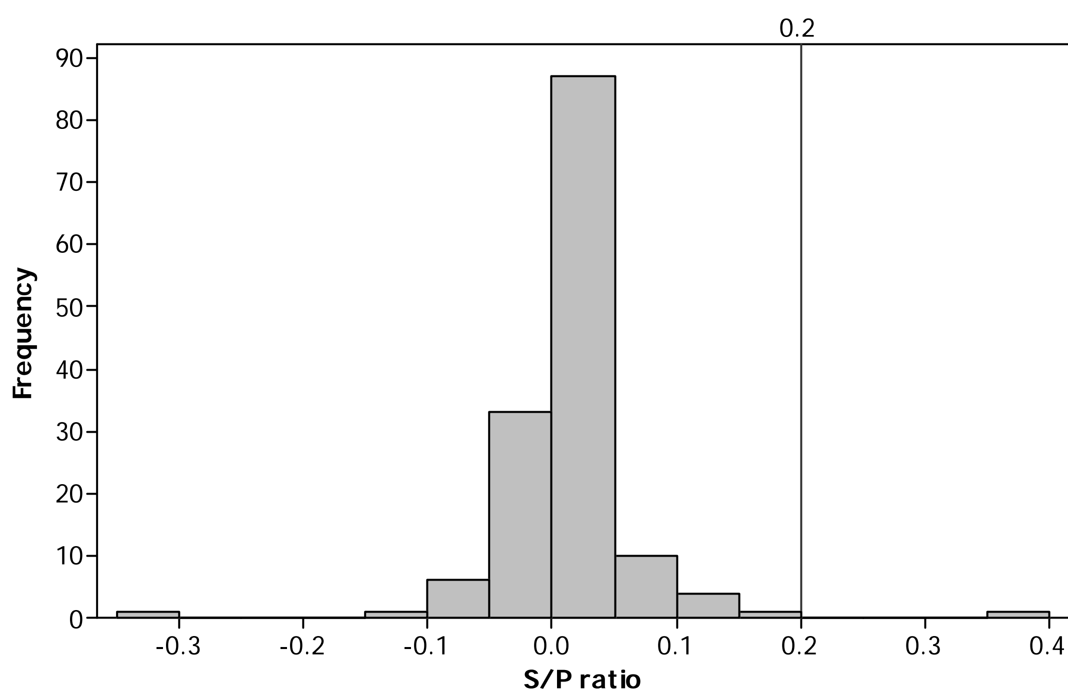

Figure 21 Diagram showed the frequency distribution of 144 vaccinated swine sera. The cut off value was indicated as the vertical line. (n = 144, mean = 0.013, S.D. = 0.057, maximum = 0.388, minimum = -0.302)

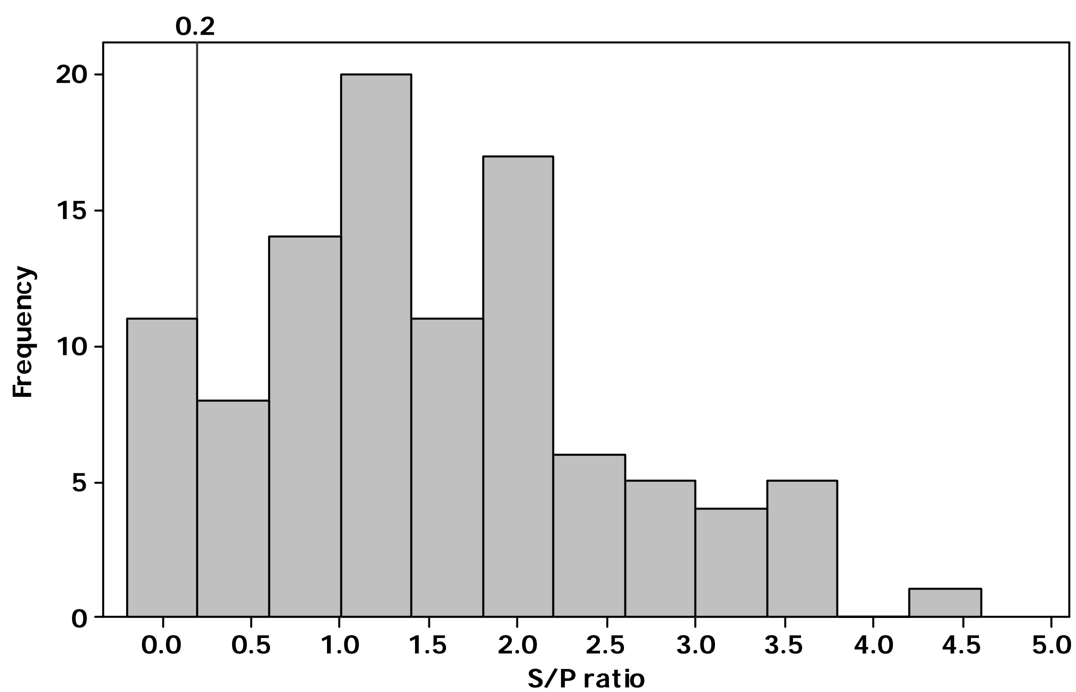

Figure 22 Showed the frequency distribution of the S/P ratio from 102 naturally-FMD infected sera. The cut off value was indicated as the vertical line. (n = 102, S.D. = 1.00, mean = 1.50, maximum = 4.28, minimum = -0.01)

Table 13 Summary of the parameters of all group of swine sera using KU-ELISA 3AB test kit

	Number	Mean	S.D.	Specificity (%)		Sensitivity (%) (positive/total)	
				Mean+3SD	Mean+4SD	Mean+3SD	Mean+4SD
Noninfected sera	195	0.05	0.05	98.97 (2/195) [a]	100 (0/195) [a]	-	-
Vaccinated sera	144	0.057	0.013	99.30 (1/144) [a]	99.30 (1/144) [a]	-	-
Infected sera	102	1.50	1.00	-	-	90.09 (91/101) [a]	87.12 (88/101) [a]

[a] No. of positive results / No. of sera analyses

Table 14 Comparison among KU-ELISA 3AB and commercial test kits (ELISA-A and ELISA-B). Seventy nine infected swine sera samples were used for the agreement among tests. The kappa was 0.479 between KU-ELISA 3AB and ELISA-A, 0.835 between KU-ELISA 3AB and ELISA-B and 0.367 between ELISA-A and ELISA-B.

a.

		KU-ELISA 3AB		Total
		Positive	Negative	
ELISA-A[a]	Positive	49	0	49
	Ambiguous	7	1	8
	Negative	13	9	22
		69	10	79

b.

		KU-ELISA 3AB		Total
		Positive	Negative	
ELISA-B[b]	Positive	67	1	68
	negative	2	9	11
		69	10	79

c.

		ELISA-B[b]		Total
		Positive	Negative	
ELISA-A[a]	Positive	48	1	49
	Ambiguous	6	2	8
	Negative	14	8	22
		68	11	79

a = CHEKIT® FMD-3ABC po (Bommeli Diagnostic, Switzerland)

b = UBI® FMDV NS NIA (United Biomefical Inc., New York, USA)

3.4 Establishing S/P Ratio for Bovine Sera

One hundred and twenty one bovine sera FMD free farm for more than 5 years was used to determine the frequency distribution of the KU-ELISA 3AB test kit as indicated in figure 23. Frequency distribution of the S/P ratio of negative bovine sera was normally distributed by having mean = 0.09, S.D. = 0.06, maximum = 0.20 and minimum = -0.06. Usually, the S/P ratio 0.27, equivalent to mean + 3S.D, which gave 100% specificity, was used. By the KU-ELISA 3AB test kit, the maximum value of negative bovine sera was 0.20 which was equivalent to mean +2S.D. (0.21). Moreover, the minimum S/P ratio of 71 naturally infected bovine sera was 0.21. The value of mean +2S.D. was determined to be the cut off value for bovine sera because it gave 100% sensitivity and specificity (Figure 23).

Seventy five bovine sera were used to study the agreement between the KU-ELISA 3AB and a commercial test kit (Ceditest® FMDV NS, designated as ELISA-C). The results can be divided into two groups. First group, sixty four infected-bovine sera collected after 14 months of FMD outbreak showed the S/P ratio of 76.92% (50/64) by KU-ELISA 3AB test kit and 96.88% (62/64) by ELISA-C test kit. Second group, eleven calves born from the first group aging between 7 to 13 months, gave the negative results for both KU-ELISA 3AB and ELISA-C test kits. The kappa statistic value between the two tests was 0.671 which interpreted as the substantial agreement (Table 15).

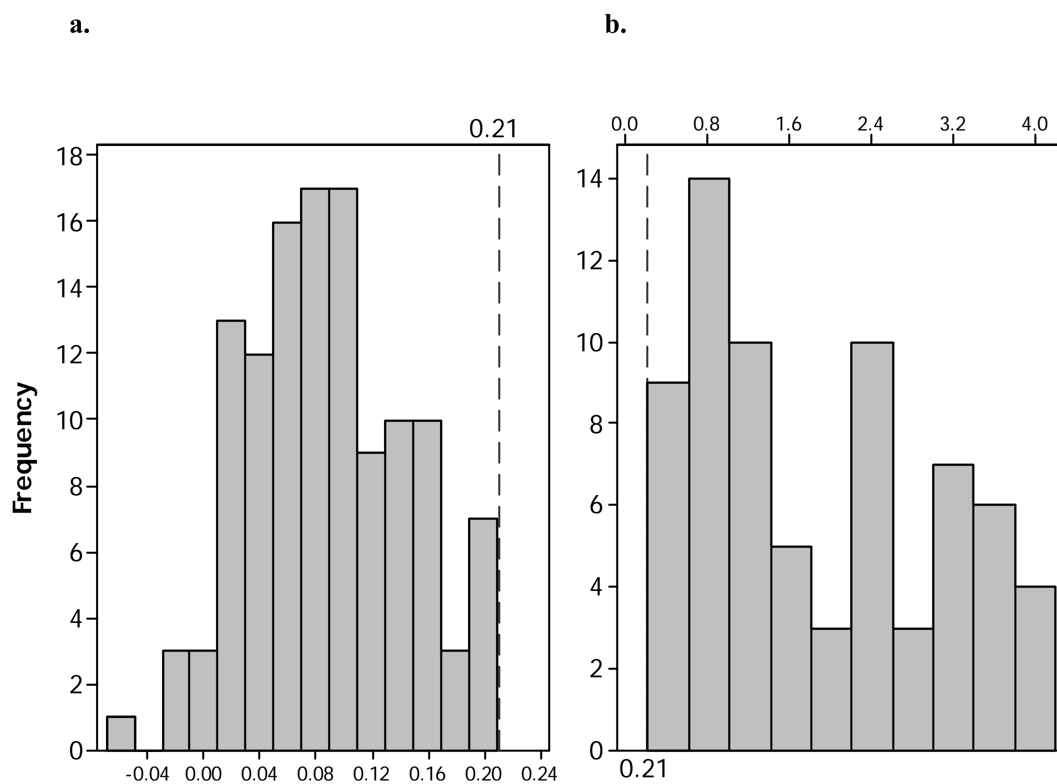

Figure 23 Frequency distribution of S/P ratio from non-infected (a) and infected bovine sera (b) tested with KU- ELISA 3AB test kit. The cut off value was indicated as the vertical line. (n = 121, mean = 0.09, S.D. = 0.06, maximum = 0.20 and minimum = -0.06 from non-infected bovine sera and n = 71, mean = 1.87, S.D. = 1.14, maximum = 4.11 and minimum = 0.21 from infected bovine sera)

Table 15 The comparison between KU-ELISA 3AB and commercial test kit (ELISA-C). The kappa was 0.671.

		KU-ELISA 3AB		Total
		Positive	Negative	
ELISA-C[a]	Positive	53	9	62
	negative	0	13	13
		53	22	75

a = Ceditest® FMDV NS (Lelystad, Netherland)

4. <u>3AB Monoclonal Antibodies</u>

The 3AB recombinant protein from *E.coli* was purified and used to immunize a Balb/C mouse for the monoclonal antibody production. The 5 clones of hybridoma were reacted with KU-ELISA 3AB test kit and were designated as 1-8C, 2-9A, 2-12F, 1-12B and 4-11A. Subsequently, the supernatant were used to test for the protein specificity and isotype identify. However, two clones out of the five clones, designated as 1-12B and 4-11A, reacted with only UBI® FMDV NS NIA test kit which was produced by using 3B peptide. Therefore, these two clones were specific for 3B peptide and another three clones were specified for 3A peptide (Table16). The light chain of all clones was kappa, and the heavy chain of all clones was IgG1 with the exception of clones 2-12F and 1-12B were IgG2 and IgM, respectively. All MAbs were used to perform IPMA technique using BHK-21-infected cells with serotype O, A and Asia1. The results showed the cross reactivity of all serotypes (Figure 24 and Table 16).

Table 16 Evaluation of 5 monoclonal antibodies by KU-ELISA 3AB, UBI® FMDV NS NIA, Mouse MonoAb ID KIT HRP (ZYMED®) and IPMA

MAb No	KU- ELISA 3AB	UBI® FMDV NS NIA	IPMA[a]	Antibody Isotype	
				Heavy chain	Light chain
1-8C	+	-	+	Gamma1	kappa
1-12B	+	+	+	IgM	kappa
2-9A	+	-	+	Gamma1	kappa
2-12F	+	-	+	Gamma2a	kappa
4-11A	+	+	+	Gamma1	kappa

+ = Positive result, - = negative result

a = BHK-21 cells were infected by serotype O, A and Asia1.

a.

b.

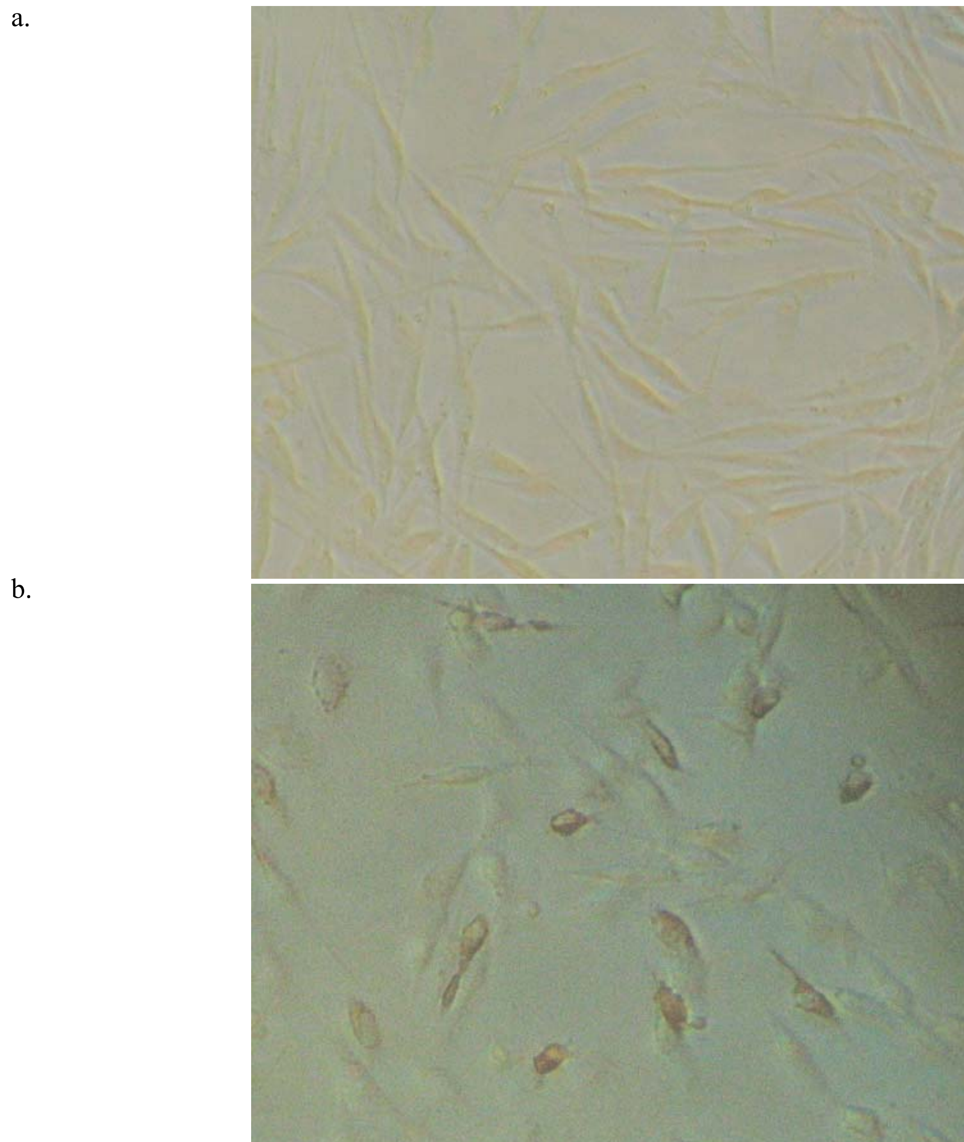

Figure 24 showed the IPMA results of non-infected (a) and FMDV serotype O-infected BHK21 (b) cells using monoclonal antibody. The non-infected BHK-21 cells showed the negative result. The infected-BHK-21 cells showed the dark brown color in the cytoplasm of the infected cells.

DISCUSSION

1. Phylogenetic tree

The difference of nucleotide sequences has been investigated for genetic variant of FMDV isolates in Thailand. In this report, the 190 bp (nucleotide 361 to 550 of VP1 gene) comparing with nucleotide 469 to 639 and complete gene (Stram *et al.,* 1995; Marquardt and Haas, 1998; Tsai *et al*., 2000; Samuel and Knowles, 2001) of FMDV isolates in Thailand had the similar patterns of dendrogram and divergence percentage. This result showed that the nucleotide position 361 to 550 of VP1 gene can be used to construct the phylogenetic tree of FMDV isolate in Thailand.

According to phylogenetic studies, the FMDVs in Thailand had high genetic variant which could be classified into lineage and sublineage based on nucleotide sequences. According to Samuel and Knowles (2001) FMDV serotype O can be divided into eight distinct genetic lineages, however, the FMDV serotype O isolated in Thailand during 1998 to 2005 were belonged to 3 topotypes (SEA, Pan-Asia strain in ME-SA and Cathay). This result was different from the previous report (Huang *et al.,* 2001) which showed that viruses isolated from Thailand in 1980 were SEA topotype. This study also showed that the SEA topotype was an endemic topotype of Thailand as reported by Huang *et al.,* 2001 and this topotype had high nucleotide and amino acid sequence variation. The viruses of Pan Asia isolated in the Northern and Northeastern part of Thailand during 1999 to 2001 had 3.8 % of divergence percentage among viruses isolated in Thailand and viruses isolated in Japan and neighboring countries (Tsai *et al.,* 2000; Kweon *et al.,* 2002; Mason *et al*., 2003). As the result, the Pan Asia viruses in Thailand had the common ancestor with these countries. The two new isolates in 2005 gave the similarity of nucleotide sequence with Cathay topotype which is principally found in Hong Kong and China (Samuel and Knowles, 2001). Both of these viruses had a deletion of nucleotide between residues 93 to 102 of 3A protein but not found between residues 133 to 143 as initial report in virus isolated in Taiwan in 1997 (Beard and Mason, 2000). The deleted region concerned infectivity in swine but not affect cattle (Beard and Mason, 2000; Knowles *et al*., 2001; Pacheco *et al.,* 2003). The O/189

virus, the vaccine strain of Thailand, had 19.4% divergence percentage compared with other topotypes including all viruses isolated from Thailand. However, there was the serological relationship (r-value) between vaccine strain and field strains during 1990 to 2002 (Linchongsubongkoch, 2003).

SEA topotype had the widest range of divergence percentage based on dendrogram (Figure 10) which could be divided into 3 lineages (I-III). Lineage I contained viruses isolated during 1998 to 2005 including virus isolated in 1980 (Huang *et al.,* 2001). Moreover, they could be clustered into 6 sublineages (IA-IF). Although, viruses isolated in Thailand during 1980 to 2005 were in this lineage but they were in difference sublineage. For example, the viruses isolated in 1980 and 2005 are separated in different sublineages. Thus, the emergence of sublineage may relate to the time of outbreak. Lineage II contained viruses isolated during 1998 to 2001 which divided into 4 sublineages. Lineage III could be comprised of 3 sublineages. Some sublineages showed geographical related to the outbreak. For example, sublineage IIA found to be viruses that circulated in the Southern part of Thailand in 2000, whereas viruses in sublineage IID circulated in the Northeastern part. However, these viruses were closely related due to the divergence percentage of each sublineage is less than 5%.

Substitution of amino acid residues at antigenic region is important for the immunological response. Alternative amino acid residues were not found in critical residue (144, 147, 148 and 154) of most of the viruses. In this study, the N-terminal part of RGD between field strains and O/189 strain showed substitution in many residues, however, there was no serological different between field strains and vaccine strain in Thailand (Linchongsubongkoch, 2003). These residues were located at the G-H loop which was a major immunogenic region of FMDV (Bittle *et al*., 1982; Robertson *et al*., 1983; Meloen and Barteling, 1986; Thomas *et al*., 1988a; Acharya *et al*., 1989; Collen *et al*., 1991; Martinez *et al*., 1991; Van Lierop *et al*., 1992, 1994; Mateu *et al.*, 1994; Rieder *et al.*, 1994; Dunn *et al.*, 1998; Sanz-Parra *et al.*, 1998; Verdaquer *et al*., 1998; Blanco *et al*., 2000; Filgueira *et al*., 2000; Marquardt *et al*., 2000; Marquardt and Freiberg, 2000).

Variation of field strains of serotype A was revealed by phylogenetic tree and divergence percentage. The percentage of difference among field strains is less than 10.3% as the viruses were in the same lineage. The divergence percentages of vaccine strains A98/A22, A/100/SKNK/97 and A118/87 calculated with filed strain were 11.5-16.7%, 1.1-7.3% and 7.3–10.9%, respectively. The serological relationship was used to select the vaccine strain. The A/NPT/87 was used to be the vaccine strain before 1997, then the A/SakonNakhon/97 isolated from Sakon Nakhon province in 1997 was selected for vaccine strain. Subsequently, A118/87 was used for the vaccine production since 2001 (Linchongsubongkoch, 2003). The viruses of serotype A has been divided into 10 lineages, designated as I-X (Tosh *et al.,* 2002). The viruses isolated in Thailand during 1998 to 2003 were clustered into lineage IX including 3 vaccine strains. Furthermore, these viruses can be subdivided into 3 sublineages (A-C). SublineageA had been found since 1998 to 2002 including vaccine strain (A/100/SKNK/97) and distributed in every parts of Thailand. The majority of amino acid residues are found to be more similar with A/100/SKNK/97 than the other two vaccine strains. SublineageB was found in viruses isolated during 2001 to 2002 when A118/87 strain has been used for vaccination. Only two viruses isolated in the Northeastern part was the member of sublineageC. According to these results, the present of each sublineage may be related to virus selection for vaccination. SerotypeA had several substitutions at critical residues which may be concerned to the variation of serological relationship. Viruses in the same area but different year of outbreak were in the difference sublineage. For instance, the outbreak viruses at Nakhon Pathom province in 2001 and 2003 were classified in the sublineageI and II, respectively. Moreover, the virus isolated in 2003 was closely related with viruses isolated from the Northeastern part in 2001.

Sanyal *et al.,* (2004) had divided serotype Asia1 into 6 lineages (I-X). Two isolates of serotype Asia1 used in this study fell into lineage II. Both viruses were closely related with Asia/TAI/1/98 isolated in 1998. These viruses had the identical amino acid residue. The serological relationship study showed that there was no significantly change in the antigenic characteristic among field strains isolated during 1990-1997 and vaccine strain (Asia1/PBR/85) (Linchongsubongkoch, 2003).

Viruses of each lineages and sublineages caused outbreak from time to time in the same geographical areas which may be the results of animal transportation or viral mutation in infected animals or persistent animals. Viruses have been shown to be long alive and found to have high mutation rate in persistent animals (Gebauer *et al.,* 1988; Dawe *et al.,* 1994).

2. Synthetic Peptide Vaccine

Synthetic peptide vaccine is a type of vaccine which has been developed for disease protection in several diseases including FMD (Bittle *et al*., 1982; Van Lierop *et al*., 1992, 1994; Zamorano, *et al.*, 1995; Volpina, *et al.*, 1996, 1999; Wang *et al*., 2001, 2002; Rodriquez *et al.*, 2003). The synthetic peptide vaccine could induce neutralizing antibody and antibody against VP1 protein in swine. Furthermore, the synthetic peptide vaccine induced higher neutralizing antibody titer than commercial vaccine (trivalent strains). The high SN titers of 2.61-2.88 were found after 2 weeks post 2nd immunization. The level of SN titer that gave 100% protection against FMDV serotype O in swine was 1.89 (Patanaprasith *et al*., 2000). According to these data, the synthetic peptide vaccine may induce 100% protection against FMDV. Furthermore, the previous reports showed that single inoculation of the synthetic peptide elicited neutralizing antibody to protect of animals against subsequent challenge with FMDV (Bittle *et al*., 1982; Zamorano *et al.,* 1995). According to the previous study, the synthetic peptide vaccine has been reported to give cross antibody to protect against several strains of FMDV (Manisa, Campos and O1 Taiwan), although the protective levels against these strains were difference (Wang *et al.,* 2002). In this study, the synthetic peptide vaccine also induced cross antibody against FMDV strain O/189. The critical amino acid residues of all FMDV serotype O isolated from Thailand during 1998 to 2005 were similar with the synthetic peptide vaccine. According to these results, the synthetic peptide vaccine might give the cross protection against all FMDV serotype O in Thailand. However, a Pan Asia isolate (O/1/CR/99) had three changes at amino residue positions 139 and 140 and 158. The SEA topotype isolates of Thailand had high variant in upstream (N-terminal) of receptor (RGD motif). The mutations of these strains occurred out side the critical amino acid residues region which should not affect the efficacy of the protection of the synthetic peptide vaccine.

For the challenge experiment, lesions were not seen in either control or vaccinated groups. By using UBI® FMDV NS EIA test kit, serum from both groups also gave the negative results. This meant that there was no virus replication in these pigs because the challenge virus may have some mutation during viral passage in culture cells which may not infect or replication in the animal. It has been shown that the mutation at RGD motif was essential for the virus to interact with integrin receptor molecules expressed in BHK cells (Saiz *et al.,* 2002).

Moreover, this synthetic peptide vaccine could provide some cross protection against serotype Asia1 but not serotype A (A/Sakonnakhon/97), because it gave low neutralizing antibody titers (0.36 to 1.2) against serotype Asia1. The SN titer that gave 100 % protection against serotype Asia1 was 1.41 (Patanaprasith *et al.,* 2000). Thus, the synthetic peptide vaccine may give the protection against serotype Asia1 less than 100%. According to the data, the synthetic peptide vaccine may be a useful tool for control the FMDV in pigs.

3. <u>3AB Enzymed Linked Immunosorbent Assay</u>

The specificity of the KU-ELISA 3AB test kit with the swine sera from imported swine and vaccinated sows was 98.97-99.30% using mean S/P ratio +3S.D (0.2). The sensitivity was 90.09% from 101 sera of infected swine collected from 3 weeks after initial outbreak. Chung *et al. (*2002) reported that all pigs in infected populations had positive reaction between 3 and 9 weeks after the observation of the first lesion. The expressed 3AB in this study was weakly reacting with antibodies from vaccinated sows which are received at least 10 vaccinations. One hundred percent of specificity and sensitivity for bovine sera was set at cut off value by mean S/P ratio+2S.D. (2.1). Antibodies of non-infected bovine sera receiving vaccination from the FMD free farm were negative. The KU-ELISA 3AB test kit gave the positive result of 76.92% (50/64) of bovine sera 14 months after infection. The infected animals had been reported the presence of the antibodies against 3AB protein between day 8 to more than 3 years after infection (Sorensen *et al.*, 1998; Chung *et al*., 2002). The calf sera collected from 7-13 months after born from infected bovine gave negative results to KU-ELISA 3AB test kit. The results show no maternal immunity between 7-13 months after birth.

The KU-ELISA 3AB test kit was evaluated for the agreement test by comparison with commercial test kits. The agreement between KU-ELISA 3AB test kit and ELISA-B (UBI® FMDV NS EIA) using swine sera was perfect, however, the agreement between KU-ELISA 3AB test kit and ELISA-A (CHEKIT® FMD 3ABC) was moderate. The kappa between ELSIA-A and -B was also low (0.367). Commercial test kits were used to evaluate and compare with CHEKIT FMDV 3ABC test kit showed 23% sensitivity and 98% specificity (Bronsvoort *et al.*, 2004) and the kappa between CHEKIT® FMD 3ABC and UBI ® FMDV NS EIA test kits was 0.583 (Lee, *et al.,* 2004). The kappa between KU-ELISA 3AB test kit and ELISA-C (Ceditest® FMDV NA) using 75 bovine sera was 0.671 which showed the substantial agreement. The advantage of KU-ELISA 3AB test kit was the versatility of the kit that can be used with both swine and bovine samples. Moreover, it may be use to detect the persistently infected animals and differentiate infected from vaccinated animals. The differences of specificity and sensitivity of between KU-ELISA 3AB test kit and other test kit especially ELISA-A depend on (i) sample size which may be too small, (ii) experimentally infected groups gave specificity and sensitivity rather than naturally infected populations, (iii) disease stages, (iv) sub populations and between populations which depend on the distribution of influent covariates (e.g., age, breeds and management system) (Huang *et al.,* 2002; Bronsvoort *et al.,* 2004; Clavijo *et al.,* 2004) and (v) different in the protocols of different test kit for setting cut off value and experimental design (Kweon *et al.,* 2003. Moreover, the 3A antibodies may present in some sera samples of vaccinated animals (Shen *et al.,* 1999)

4. <u>3AB Monoclonal Antibodies</u>

The 5 clones of MAb can react with KU-ELISA 3AB test kit which bases on recombinant 3AB protein, but 2 clones out of 5 clones react with UBI® FMDV NS NIA. Hence, these 2 MAbs (1-12B and 4-11A) should react with epitope located in 3B protein and another 3 clones (1-8C, 2-9A and 4-11A) should react with epitope located in 3A protein. The heavy chain of MAb (1-12B) was found to be mu (μ) which was isotype of IgM due to cross reaction of the second antibody to light chain. These MAbs can detect FMDV-infected cells (BHK21) of all 3

serotypes (O, A and Asia1) by IPMA technique because 3AB protein were highly conserved among three serotypes of FMDV in Thailand.

CONCLUSION

The circulated FMDVs in Thailand during 1998 to 2005 were highly genetic variant based on nucleotide 361 to 550 of VP1 gene. Serotype O in Thailand was found to be SEA, Pan Asia strain in ME-SA and Cathay topotypes. The SEA topotype caused endemically outbreak in Thailand and can be divided into 3 lineages and several sublineages. The Pan Asia strain in ME-SA topotype was found to cause several outbreaks during 1999 to 2001 and the Cathay topotype was firstly recognized in early 2005. The synthetic peptide vaccine could induce neutralizing antibodies against the O/189 vaccine strain of Thailand which were found to induce higher titer of antibody than commercial vaccine (trivalent strains). The KU-ELISA 3AB test kit was stable for at least 2 months when kept in dark and cool place. The KU-ELISA 3AB test was shown to be highly sensitive and specific and can discriminate between infected and non-infected animals. The KU-ELISA 3AB test could be a very useful tool in control and eradication of foot-and-mouth disease in Thailand. The virus epidemiology, the selection of virus vaccine strain and vaccine type, the sensitive and specific diagnostic tools and quarantine protocol are important for FMD control in Thailand.

LITERATURE CITED

Acharya, R., E. Fry, D. Stuart, G. Fox, D. Rowlands and F. Brown. 1989. The three dimensional structure of foot and mouth diseae virus at 2.9 A° resolution. **Nature**. 37: 709-716.

Alexandersen, Z. Zhang, A.I. Donaldson and A.J.M. Garland. 2003. The pathogenesis and diagnosis of foot and mouth disease. **J. Comp. Path.** 129: 1-36.

Amaral-Doel, C.M.F., N.E. Owen, N.P. Ferris, R. P. Kitching and T.R. Doel. 1993. Detection of foot and mouth disease viral sequences in clinical specimens and ethyleneimine inactivated preparation by the polymerase chain reaction. **Vaccine**. 11: 415-421.

Armstrong, R.M., S.J. Cox, N. Aggarwal, D.J. Mackay, P.R. Davies, P.A. Hamblin, P. Dani, P.V. Barnett and D.J. Paton. 2005. Detection of antibody to the foot and mouth disease virus (FMDV) non structural polyprotein 3ABC in sheep by ELISA**. J. Virol. Methods**. 15: 153-163.

Barnett, P.V., E.J. Ouldridge, D.J. Rowlands, F. Brown and N.R. Parry. 1989. Neutraling epitopes of type O foot-and-mouth disease virus identification and characterization of three functionally independent, conformational sites. **J.Virol**. 70: 1483-1491.

_____, A.R. Samuel, L. Pullen, D. Ansell, R.N. Butcher and R.M.E. Parkhouse. 1998. Monoclonal antibodies, against O1 serotype foot-and-mouth disease virus, from a natural bovine host , recognize similar antigenic features to those defined by the mouse. **J.Gen.Virol**. 79:1687-1697.

Bastos, A.D.S., H.J. Bertschinger, C. Cordel, C. De, W.J. van Vuuren, D. Keet, R.G. Bengis, D. G. Grobler and G. R. Thomson. 1999. Possibility of sexual transmission of foot and mouth disease from African buffalo to cattle. **Vet. Rec**. 17: 77-79.

Baxt, B., D.O. Morgan, B.H. Robertson and C.A. Timpone. 1984. Epitopes on foot-and-mouth disease virus outer capsid protein VP1 involved in neutralization and cell attachment. **J. Virol.** 51: 298-305.

_____, V. Vakharia, D.M. Moore, A.J. Franke and D.O. Morgan. 1989. Analysis of neutralizing antigenic site on the surface of type A12 foot and mouth disease virus. **J. Virol.** 63: 2143-2151.

Bayry, J., K. Prabhudas, S. Gopalakrishna, P. K. Patil, C. Ramakrishna, L. D. Misra and V.V.S. Suryanarayana. 1999. Protective immune response to 16 kDa immunoreactive recombinant protein encoding the C-terminal VP1 portion of foot and mouth disease virus type Asia1. **Microbiol. Immunol**. 43: 765-771.

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

Beck, E. and K. Strohmaier. 1987. Subtyping of European foot and mouth disease virus strains by nucleotide sequence determination. **J. Virol**. 61: 1621-1629.

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

Bittle, J.L., R.A. Houghten, H. Alexander, T.M. Shinnick, J.G. Sutcliffe and R.A. Lerner, D.J. Rawlands and F. Brown. 1982. Protection against foot and mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. **Nature**. 298: 30-33.

Blanco, E., K. McCullough, A. Summerfield, J. Fiorini, D. Andreu, C.Chiva, E. Borras, P. Barnett and F. Sobrino. 2000. Interspecies major histocompatibility complex-restricted Th cell epitope on foot and mouth disease virus capsid protein VP4. **J. Virol**. 74: 4902-4907.

Bronsvoort, B.M.D.C., K.J. Sorensen, J. Anderson, A. Corteyn, V.N. Tanya, R.P. Kitching and K.L. Morgan. 2004. Comparison of two 3ABC enzyme linked immunosorbent assays for diagnosis of multiple serotype foot and mouth disease in a cattle population in an area of endemicity. **J. Clin. Microbiol**. 42: 2108-2114.

Bruderer, U., H. Swam, B. Hass, N. Visser, E. Brocchi, S. Grazioli, J.J. Esterhuysen, W. Vosloo, M. Forsyth, N. Aggarwal, S. Cox, R. Armstrong and J. Anderson. 2004. Differentiating infection from vaccination in foot-and-mouth-disease: evaluation of an ELISA based on recombinant 3ABC. **Vet. Microbiol**. 101: 187-197.

Callens, M. and K. De Clercq. 1997. Differentiation of the seven serotypes of foot and mouth disease virus by reverse transcriptase polymerase chain reaction. **J. Virol. Methods**. 67: 35-44.

Capozzo, A.V.E., D.J. Burke, J.W. Fox, I.E. Bergmann, J.L. La Torre and P.R. Grigera. 2002. Expression of foot and mouth disease virus non structural polypeptide 3ABC induces histone H3 cleavage in BHK21 cells. **Virus Res.** 90: 91-99.

Carrillo, C., E.R. Tulman, G. Delhon, Z. Lu, A. Carreno, A. Vagnozzi, G.F. Kutish and D.L. Rock. 2005. Comparative genomics of foot-and-mouth disease virus. **J. Virol.** 79: 6487-6504.

_____, C., A. Wigdorovitz, J.C. Oliveros, P.I. Zamorano, A.M. Sadir, N. Gomez, J. Salinas, J.M. Escribano and M.V. Borca. 1998. Protective immune response to foot and mouth disease virus with VP1 expressed in transgenic plants. **J. Virol.** 72: 1688-1690.

Chaisrisongkram, W. 1993. An overview of foot and mouth disease control in Thailand, pp. 23-25 . *In* J.W. copland, L.J. Gleeson and C. Chamnanpood, eds. **Diagnosis and Epidemiology of Foot and Mouth Disease in Southeast Asia.** Lampang, Thailand.

Chenard, G., K. Miedema, P. Moonen, R.S. Schrijver and A. Dekker. 2003. A solid phase blocking ELISA for detection of type O foot and mouth disease virus antibodies suitable for mass serology. **J. Virol. Methods**. 107: 89-98.

Cheung, A., J. De Lamarter, S. Weiss and H. Kupper. 1983. Comparison of the major antigenic determinants of different serotypes of foot and mouth disease virus. **J. Virol**. 48: 451-459.

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

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

_____, P.Wright and P. Kitching. 2004b. Developments in diagnostic techniques for differentiating infection from vaccination in foot and mouth disease. **Vet. J**. 167: 9-22.

Collen, T., R. Dimarchi and T.R. Doel. 1991. A T cell epitope in VP1 of foot and mouth disease virus is immunodominant for vaccinated cattle. **J. Immunol.** 146: 749-755.

Collins, R.A., L.S. Ko, K.Y. Fung, L.T. Lau, J. Xing and A.C.H. Yu. 2002. A method to detect major serotypes of foot-and-mouth disease virus. **Biochem. Biophys. Res. Com.** 297: 267-274.

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

Crowther, J.R., S. Farias, W.C. Carpenter and A.R. Samuel. 1993. Identification of a fifth neutralizable site on type O foot and mouth disease virus following characterization of single and quintuple monoclonal antibody escape mutants. **J. Gen.Virol.** 74: 1547-1553.

Davies, G. 2002. Foot and mouth disease. **Res. Vet. Sci**. 73: 195-199.

Dawe, P.S., K. Sorensen, N.P. Ferris, I. T. R. f, R.M. Armstrong and N.J. Knowles. 1994. Experimental transmission of foot and mouth disease virus carrier African buffalo (Syncerus caffer) to cattle in Zimbabwe. **Vet. Rec**. 26: 211215.

Devaney, M.A., V.N. Vakharia, R.E. Lloyd, E. Ehrenfeld and M.J. Grubman. 1988. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. **J. Virol**. 62: 4407-4409.

Domingo, E., E. Baranowski, C. Escarmis and F. Sobrino. 2002. Foot and mouth disease virus. **Comp. Immun. Microbiol. Infect. Dis.** 25: 297-308.

_____, C. Escarmis, E. Baranowski, C. M. Ruiz-Jarabo, E. Carrillo, J.I. Nunez and F. Sobrino. 2003. Evolution of foot and mouth disease virus. **Virus Res**. 91: 47-63.

Donnelly, M.L.L., L.E. Hughes, G. Luke, H. Mendoza, E.T. Dam, D. Gani and M.D. Ryan. 2001. The ‘cleavage’ activities of foot and mouth disease virus 2A site-directed mutants and naturally occurring ‘2A-like’ sequences. **J. Gen. Virol**. 82: 1027-1041.

Duchesne, M., T. Cartwright, A. Crespo, F. Boucher and A. Folloured. 1984. Localization of a neutralization epitope of foot and mouth disease virus using neutralizing monoclonal antibodies. **J. Gen. Virol**. 65: 1559-1566.

Dunn, C.S., A.R. Samuel, L.A. Pullen and J. Anderson. 1998. The biological relevance of virus neutralisation sites for virulence and vaccine protection in the guinea pig model of foot and mouth disease. **Virology.** 247: 51-61.

Dus Santos, M.J., A. Wigdorovitz, K. Trono, R.D. Rios, P.M. Franzone, F. Gil, J. Moreno, C. Carrillo, J.M. Escribano and M.V. Borca. 2001. A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. **Vaccine.** 2968: 1-7.

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

Ferris, N.P. and M. Dawson. 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot and mouth and swine vesicular diseases. **Vet. Microbiol**. 16: 201-209.

Filgueira, M.P., A. Wigdoroyitz, A. Romera, P. Zamorano, M.V. Borca and A.M. Sadir. 2000. Detection and characterization of functional T-cell epitopes on the structural proteins VP2, VP3, and VP4 of foot and mouth disease virus O1 campos. **Virology.** 271: 234-239.

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

Fox, G., N.R. Parry, P.V. Barnett, B. McGinn, D.J. Rowlands and F. Brown. 1989. The cell attachment site on foot and mouth disease virus includes the amino acid sequence RGD (Arginine-Glycine-Aspartic Acid). **J. Gen. Virol**. 70: 625-637.

Freiberg, B., M.M. Rahman and O. Marquardt. 1999. Genetical and immunological analysis of recent asian type A and O foot and mouth disease virus isolates. **Virus Gene.** 19: 167-182.

Gebauer, F., J.C. De La Torre, I. Gomes, M.G. Mateu, H. Barahona, B. Tiraboschi, I. Bergmann, P. Auge de Mello and E. Domingo. 1988. Rapid selection of genetic and antigenic variants of foot and mouth disease virus during persistence in cattle. **J. Virol**. 62: 2041-2049.

Golde, W.T., J.M. Pacheco, H. Duque, T. Doel, B. Pemfold, G.S. Ferman, D.R. Gregg and L.L. Rodriquez. 2005. Vaccination against foot and mouth disease virus confes complete clinical protection in 7 days and partial in 4 days: use in emergency outbreak response. **Vaccine**. 23: 5775-5782.

Grubman, M.J., M. Zellner and J. Wagner. 1987. Antigenic comparison of the polypeptides of foot and mouth disease virus serotypes and other picornaviruses. **Virology.** 158: 133-140.

Gurumurthy, C.B., A. Sanyal, R. Venkataramanan, C. Tosh, M. George and D. Hemadri. 2002. Genetic diversity in the VP1 gene foot and mouth disease virus serotype Asia1. **Arch. Virol.** 147: 85-102.

Hamblin, C., I.T.R. Barnett and R.S. Hedger. 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot and mouth disease virus. **J. Immunol. Methods**. 93: 115-121.

Hofmann, M.A., B. Thur, L. Liu, M. Gerber, P. Stettler, C. Moser and S. Bossy. 2000. Rescue of infectious classical swine fever and foot and mouth disease virus by RNA transfection and virus detection by RT-PCR after extended storage of samples in Trizol®. **J. Virol Methods**. 87: 29-39.

Hohlich, B.J., K.H. Wiesmuller, T. Schlapp, B. Haas, E. Pfaff and A. Saalmuller. 2003. Identification of foot-and-mouth disease virus-specific linear B-cell epitope to differentiate between infected and vaccinated cattle. **J. Virol**. 77: 8633-8639.

Huang, C.C, F. Lee, W.J. Tu, S.H. Lee, T.S. Huang, Y.L. Lin, M.H. Jong and S.Y. Lin. 2002. Anti-3AB antibodies in the Chinese yellow cattle infected by the O/Taiwan/99 foot-and-mouth disease virus. **Vet. Microbiol**. 84: 317-326.

_____, Y.L. Lin, T.S. Huang, W.J. Tu, S.H. Lee, M.H. Jong and S.Y. Lin. 2001. Molecular characterization of foot and mouth disease virus isolated from ruminants in Taiwan in 1999-2000. **Vet. Microbiol**. 81: 193-205.

Jacobson, R.H. 1998. Validation of serological assays for diagnosis of infectious disease. **Rev. Sci. Tech. Off. Int. Epizoot.** 17: 469-486.

Karber, G. 1931. Archive experimental pathology. **Pharmak**. 162: 480.

Kitson, J.D.A., D. MaCahon and G.J. Belsham. 1990. Sequence analysis of monoclonal antibody resistant mutants of type O foot and mouth disease virus; Evidence for the involvement of the three surface exposed capsid proteins in four antigenic sites. **Virology.** 179: 26-34.

Knowles, N.J., P.R. Davies, T. Henry, V. O'Donnell, J.M. Pacheco and P.W. Mason. 2001. Emergence in Asia of foot and mouth disease viruses with altered host range: characterization of alterations in the 3A protein. **J. Virol**. 75: 1551-1556.

_____ and A.R. Samuel. 2003. Molecular epidemiology of foot-and-mouth disease virus. **Virus Res.** 91: 65-80.

_____, A.R. Samuel, P.R. Davies, R.J. Midgley and J.F. Valarcher. 2005. Pandemic Strain of foot and mouth disease virus serotype O. **Emerging. infect. Dis.** 11: 1887-1893.

Kongthon, A. 1993. Develop of laboratory diagnosis of foot and mouth disease in Thailand, pp. 87-90. In J.W. Copland, L.J. Gleeson and C. Chamnanpood, eds**. Diagnosis and epidemiology of foot and mouth disease in southeast asia**. Lampang, Thailand.

Kweon, C.H., Y.J. Ko, W.I. Kim, B.H. Hyun, H.J. Sohn, K.S. Choi and J.H. Shin. 2002. Molecular characterization of foot and mouth disease virus O/SKR/2000. **Virus Res**. 90:15-22.

_____, _____, _____, S.Y. Lee, J.J. Nah, K.N. Lee, H.J. Sohn, K.S. Choi, B.H. Hyun, S.W. Kang, Y.S. Joo and J. Lubroth. 2003. Development of a foot and mouth disease NSP ELISA and its comparison with differential diagnostic methods. **Vaccine.** 21: 1409-1414.

Lee, F., Y.L. Lin and M.H. Jong. 2004. Comparison of ELISA for the detection of porcine serum antibodies to non-structural proteins of foot-and-mouth disease virus. **J. Virol. Methods.** 116: 155-159.

Leippert, M. and E. Pfaff. 2001. Foot and mouth disease virus can utilize the C-terminal extension of coxsackievirus A9 VP1 for cell infection. **J. Gen. Virol.** 82: 1703-1711.

Lertwatcharasarakul, P. 2000. 3AB protein expression in *E.coli* for antibody detection of foot and mouth disease virus. M.S. thesis, Kasetsart University.

Li, X., H.H. Lu, S. Mueller and E. Wimmer. 2001. The C-terminal residues of poliovirus proteinase $2A^{pro}$ are critical for viral RNA replication but not for *cis* or *trans* proteolytic cleavage. **J. Gen. Virol**. 82: 397-408.

Linchongsubongkoch, W. 2003. Recent characteristic of FMD virus in Thailand. pp. s9-s11. *In* R. Thanawongnuwech and P. ingkaninum, eds. **The 11th International Symposium of the World Association of Veterinary Laboratory Diagnosticians and OIE Seminar on Biotechnology**, Bangkok, Thailand.

_____, T. Janukit, S Romlumdoan and A. Phusirimongkol. 2000. The use of molecular biology techniques for the diagnosis and epidemiological study of foot and mouth disease virus in Thailand. pp. 406-427. *In* **The 38th Kasetsart University Annual Conference**, Kasetsart University, Bangkok.

Locher, F., V.V.S. Suryanarayana and J.D. Tratschin. 1995. Rapid detection and characterization of foot and mouth disease virus by restriction enzyme and nucleotide sequence analysis of PCR products. **J. Clin. Microbiol**. 33: 440-444.

Logan, D., R Abu-Ghazaleh, W. Blakemore, S. Curry, T. Jackson, A. King, S. Lea, R. Lewis, J. Newman, N. Parry, D. Rowlands, D. Stuart and E. Fry. 1993. Structure of a major immnogenic site on foot and mouth disease virus. **Nature**. 362: 566-568.

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

Mackay, D.K.J., A.N. Bulut, T. Rendle, F. Davidson and N.P. Ferris. 2001. A solid-phase competition ELISA for measuring antibody to foot-and-mouth disease virus. **J. Virol Methods.** 97: 33-48.

Mann, J.A., and R.F. Sellers. 1989. Foot and mouth disease virus, pp 503-512. *In* Pensaert, M.B., ed. **Virus infections of cattle**. Elsevier Science Publishing Company INC., Amsterdam, Netherlands.

Marquardt, O. and B. Haas. 1998. VP1 coding sequences of recent isolates of foot and mouth disease virus types A, O and Asia1. **Virus Genes**. 16: 185-193.

_____, and B. Freiberg. 2000. Antigenic variation among foot and mouth disease virus type A field isolates of 1997-1999 from Iran. **Vet. Microb.** 74: 377-386.

_____, M.M. Rahman and B. Freiberg. 2000. Genetic and antigenic variance of foot and mouth disease virus type Asia1. **Arch. Virol.** 145: 149-157.

Martinez, M.A., J. Hernandez, M.E. Piccone, E.L. Palma, E. Domingo, N. Knowles and M.G. Mateu. 1991. Two mechanisms of antigenic diversification of foot and mouth disease virus. **Virology.** 184: 695-706.

_____, N.Verdaquer, M.G. Mateu and E. Domingo. 1997. Evolution subverting essentiality: dispensability of the cell attachment Arg-Gly-Asp motif in multiply passaged foot and mouth disease virus. **Proc. Natl. Acad. Sci. USA.** 94: 6798-6802.

Mason, P.W., M.J. Grubman, B. Baxt. 2003. Molecular basis of pathogenesis of FMDV. **Virus Res**. 91: 9-32.

Mateu, M.G., J. Hernandez, M.A. Martinez, D. Feigelstock, S. Lea, J.J. Perez, E. Giralt, D. Stuart, E.L. Palma and E. Domingo. 1994. Antigenic heterogeneity of a foot and mouth disease virus serotype in the field is mediated by very limited sequence variation at several antigenic sites. **J. Virol**. 68: 1407-1417.

_____, M.A. Martinez, L. Capucci, D. Andreu, E. Giralt, F. Sobrino, E. Brocchi and E. Domingo. 1990. A single amino acid substitution affects multiple overlapping epitopes in the major antigenic site of foot-and-mouth disease virus of serotype C. **J. Gen. Virol**. 71: 629-637.

McKenna, T.S.C., E. Rieder, J. Lubroth, T. Burrage, B. Baxt and P.W. Mason. 1996. Strategy for producing new foot and mouth disease vaccines that display complex epitopes**. J. Biotechnol.** 44: 83-89.

Meloen, R.H., J Briaire, R.J. Woortmeyer and D. Vanzaane. 1983. The main antigenic determinant detected by neutralizing monoclonal antibodies on the intact foot-and-mouth disease virus particle is absent from isolated VP1. **J. Virol**. 64: 1193-1198.

_____and S.J. Barteling. 1986. An epitope located at the C terminus of isolated VP1 of foot and mouth disease virus type O induced neutralizing activity but poor protection. **J. Gen. Virol.** 67: 289-294.

Mohapatra, J.K., A. Sanyal, D. Hemadri, C. Tosh, G.P. Sabarinath and R. Venkataramanan. 2002. Sequence and phylogenetic of L and VP1 genes of foot and mouth disease virus serotype Asia1. **Virus Res.** 87: 107-118.

_____,_____,_____,_____,_____, R.M. Kumar, R. Venkataramanan and S.K. Bandyopadhyay. 2004. Sequence variability in the structural protein-enconding region of foot and mouth disease virus serotype Asia1 field isolates. **Res. Vet. Sci.** 77: 153-161.

Moonen, P., E.V.D. Linde, G. Chenard and A. Dekker. 2004. Comparable sensitivity and specificity in three commercially available ELISAs to differentiatiate between cattle infected with or vaccinated against foot and mouth disease virus. **Vet. Microbiol**. 99: 93-101.

Neitzert, E., E. Beck, P.A. De Mello, I. Gomes and I.E. Bergmann. 1991. Expression of the Aphthovirus RNA polymerase gene in *Escherichia coli* and its use together with other bioengineered nonstructural antigens in detection of late persistent infections. **Virology.** 184: 799-804.

Pacheco, J.M., T.M. Henry, V.K. O'Donnell, J.B. Gregory and P.W. Mason. 2003. Role of nonstructural proteins 3A and 3B in host range and pathogenicity of foot-and-mouth disease virus. **J. Virol.** 77: 13017-13027.

Pattnaik, B., R. Venkataramanan, C. Tosh, A. Sanyal, D. Hemadri, A.R. Samuel, N.J. Knowles and R.P. kitching. 1998. Genetic heterogeneity of Indian field isolates of foot and mouth disease virus serotype O as revealed by partial sequencing of 1D gene. **Virus Res.** 55: 115-127.

Piatti, P.G., A. Berinstein, O.J. Lopez, M.V. Borca, F. Fernandez, A.A. Schudel and A.M. Sadir. 1991. Comparison of the immune response elicited by infectious and inactivated foot and mouth disease virus in mice. **J. Gen. Virol.** 72: 1691-1694.

Pinto, A.A. and R.S. Hedger. 1978. The detection of antibody to virus-infection associated (VIA) antigen in various species of African wildlife following natural and experimental infection with foot and mouth disease virus. **Arch. Virol.** 57: 307-314.

Polatnick J. and S.H. Wool. 1982. Localization of foot and mouth disease – RNA Synthesis on newly formed cellular smooth membranous vacuoles. **Arch. Virol**. 71: 207-215.

Porter, A.G. 1993. Picornavirus nonstructural proteins: Emerging roles in virus replication and inhibition of host cell functions. **J. Virol.** 67: 6917-6921.

Prato Murphy, M.L., R.F.Meyer, C. Mebus, A.A. Schudel and M. Rodriguez. 1994. Analysis of sites of foot and mouth disease virus persistence in carrier cattle via the polymerase chain reaction. **Arch. Virol.** 136: 299-307.

_____, M.L., M. Rodriquez, A.A. Schudel and R. F. Meyer. 1995. Localization of foot and mouth disease virus RNA in tissue culture infected cells via in situ polymerase chain reaction. **J. Virol. Methods.** 54: 173-178.

Reid, S.M., N.P. Ferris, G.H. Hutchings, A.R. Samuel and N.J. Knowles. 2000. Primary diagnosis of foot and mouth disease by reverse transcription polymerase chain reaction**. J. Virol. Methods.** 89: 167-176.

_____, M.A. Forsyth, G.H. Hutchings and N.P. Ferris. 1998. Comparison of reverse transcription polymerase chain reaction, enzyme linked immunosorbent assay and virus isolation for the routine diagnosis of foot and mouth disease. **J. Virol. Methods**. 70: 213-217.

_____, G.H. Hutchings, N.P. Ferris and K. De Clercq. 1999. Diagnosis of foot and mouth disease by RT-PCR: evaluation of primers for serotypic characterisation of viral RNA in clinical samples. **J. Virol. Methods**. 83: 113-123.

Rieder, E., B. Baxt, J. Lubroth and P.W. Mason. 1994. Vaccines prepared from chimeras of foot and mouth disease virus (FMDV) induce neutralizing antibodies and protective immunity to multiple serotypes of FMDV. **J. Virol**. 68: 7092-7098.

_____, A. Berinstein, B. Baxt, A. Kang and P.W. Mason. 1996. Propagation of an attenuated virus by design: engineering a novel receptor for a noninfectious foot and mouth disease virus. **Proc. Natl. Acad. Sci. USA**. 93: 10428-10433.

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

Robertson, B.H., D.M. Moore, M. J. Grubman and D. G. Kleid. 1983. Identification of an exposed region of the immunogenic capsid polypeptide VP1 on foot and mouth disease virus. **J. Virol**. 46: 311-316.

Robiolo, B., C. Seki, N. Fondevilla, P. Grigera, E. Scodeller, O. Periolo, J.L. Torre and N. Mattion. 2006. Analysis of the immune response to FMDV structural and non-structural proteins in cattle in Argentina by the combined use of liquid phase and 3ABC-ELISA test. **Vaccine.** 24: 997-1008.

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

Rodriquez, L.L., J. Barrera, E. Kramer, J. Lubroth, F. Brown and W.T. Golde. 2003. A synthetic peptide containing the consensus sequence of the G-H loop region of foot and mouth disease virus type O VP1 and a promiscuous T-helper epitope induces peptide-specific antibodies but fails to protect cattle against viral challenge. **Vaccine.** 21: 3751-3756.

Roeder, P.L. and P.M. Le Blanc Smith. 1987. The detection and typing of foot and mouth disease virus by enzyme linked enzyme linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. **Res. Vet. Sci.** 43: 225-232.

Saiz, J.C., A. Rodriquez, M. Gonzalesz, F. Alonso and F. Sobrino. 1992. Heterotypic lymphoproliferative response in pigs vaccinated with foot and mouth disease virus involvement of isolated capsid proteins. **J. Gen. Virol.** 73: 2601-2607.

_____, F. Sobrino and J. Dopozo. 1993. Molecular epidemiology of foot and mouth disease virus type O. **J. Gen. Virol**. 74: 2281-2285.

Saiz, M., S. Gomez, E. Martnez-Salas and F. Sobrino. 2001. Deletion or substitution of the aphtovirus 3'NCR abrogates infectivity and viral replication. **J. Gen. Virol**. 82: 93-101.

_____, J.I. Nunez, M.A. Jimenez-Clavero, E. Baranowski and F. Sobrino. 2002. Foot and mouth disease virus: biology and prospects for disease control. **Microbes and Infect.** 4: 1183-1192.

Sambrook, K., E.F. Fritsch and T. Maniants. 1989. **Molecular cloning a laboratory manual**. 2nd. Cold Spring harbor laboratory press, USA. 980 p.

Samuel, A.R. and N.J. Knowles. 2001. Foot and mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). **J. Gen. Virol**. 82: 609-621.

Sanyal, A., D. Hemadri, C. Tosh and S.K. Bandyopadhyay. 2004. Emergence of a novel subgroup within the widely circulating lineage of foot and mouth disease virus serotype Asia1 in India. **Res. Vet. Sci**. 76: 151-156.

Sanz-Parra. A, R. Blasco, F. Sobrino and V. Ley. 1998. Analysis of the B and T response in guinea pigs induced with recombinant vaccinia expressing foot and mouth disease virus structural proteins. **Arch. Virol**. 143: 389-398.

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

Siebert, P.D. and A. Chenchik. 1993. Modified acid guanidinium thiocyanate-phenol-chloroform RNA extraction method which greatly reduces DNA contamination. **Nucleic. Acids Res**. 21: 2019-2020.

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

Sobrino, F., E.L. Palma , E. Beck, M. Davila, J.C de la Torre, P. Negro, N.Villanueva, J. Ortin and E. Domingo. 1986. Fixation of mutations in the viral genome during an outbreak of foot and mouth disease: heterogeneity and rate variations. **Gene**. 50: 149-159.

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

Stram, Y, D. Chai, H.E.D. Fawzy, T. Molad, N. Meiri, M. Van-Ham, S. El-Kilani, F. Fahamy, A.A.M. Moussa and H. Yadin. 1995. Molecular epidemiology of foot and mouth disease (FMD) in Israel in 1994 and in other Middle-Eastern countries in the years 1992-1994. **Arch. Virol**. 140: 1791-1797.

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

Suryanarayana, V.V.S., S. Viswanathan, G. Ratish, P. Bist, K.Prabhudas, M.R. Gajendragad, and C. Natarajan. 1999. *E.coli* expressed proteins as diagnostic reagents for typing of foot and mouth disease virus. **Arch. Virol**. 144: 1701-1712.

Sutmoller,P., S.S. Barteling, R.C. Olascoaga and K.J. Sumption. 2003. Control and eradication of foot and mouth disease. **Virus Res**. 91:101-144.

Thomas, A.A.M., R.J. Woortmeijer, S.J. Barteling and R.H. Meloen. 1988a. Evidence for more than one important, neutralizing site on foot and mouth disease virus. **Arch. Virol**. 99: 237-242.

_____,_____, W. Puijk and S.J. Barteling. 1988b. Antigenic sites on foot and mouth disease virus type A10. **J. Virol**. 62: 2782-2789.

Thongtha, P., T. Inoue, T. Janukit, B.Nuansrichay, N.J. Knowles, S. Kamolsiripichaiporn and W. Linchongsubongkoch. 2003. Molecular epidemiological analysis of recent Thai isolates of foot and mouth disease virus type O (2001 and 2002). pp. 68-69. *In* R. Thanawongnuwech and P. ingkaninum, eds. **The 11th International Symposium of the World Association of Veterinary Laboratory Diagnosticians and OIE Seminar on Biotechnology**, Bangkok, Thailand.

Thrusfield, M. 1995. Diagnostic testing *In*: Veterinary Epidemiology, second ed. Blackwell Science Ltd., Oxford, pp. 280-282.

Tosh, C., M. Mittal, A. Sanyal, D. Hemadri and S.K. Bandyopadhyay. 2004. Molecular phylogeny of leader proteinase gene of type A of foot and mouth disease virus from India. **Arch. Virol**. 149: 523-536.

_____, A. Sanyal, D. Hemadri and R. Vankataramanan. 2002. Phylogenetic analysis of serotype A foot and mouth disease virus isolated in India between 1977 and 2000. **Arch. Virol.** 147: 493-513.

Tsai, C.P., C.H. Pan, M.Y. Liu, Y.L. Lin, C.M. Chen, T.S. Huang, I.C. Cheng, M.H. Jong and P.C. Yang. 2000. Molecular epidemiological studies on foot and mouth disease type O Taiwan viruses from the 1997 epidemic. **Vet. Microbiol.** 74: 207-216.

Usha, R., J.B. Rohill, V.E. Spall, M. Shanks, A.J. Maule, J.E. Johnson and G.P. Lomonossoff. 1993. Expression of an animal virus antigenic site on the surface of a plant virus particle. **Virology.** 197: 366-374.

Van Lierop, M. J.C., K. van Maanen, R.H. Meloen, V.P.M.G. Rutten, M.A.C. De Jong and E.J. Hansen. 1992. Proliferative lymphocyte responses to foot and mouth disease virus and three FMDV peptides after vaccination or immunization with these peptides in cattle. **Immunology.** 75: 406-413.

_____, J.M. van Noort, J.P.A. Wagenaar, V.P.M.G. Rutten, J. Langeveld, R.H. Meloen and E.J. Hensen. 1994. T cell- stimulatory fragments of foot and mouth disease virus released by mild treatment with cathepsin D. **J. Gen. Virol**. 75: 2937-2946.

Vance, L.M., N. Moscufo, M. Chow and B.A Heinz. 1997. Poliovirus 2C region functions during eccapsidation of viral RNA. **J.Virol**. 71: 8759-8765.

Vandamme, A. M. 2002. Virus evolution and molecular epidemiology. **Virus Res**. 85: 1-3.

Vangrysperre, W. and K. De Clercq. 1996. Rapid and sensitive polymerase chain reaction based detection and typing of foot and mouth disease virus in clinical samples and cell culture isolates, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. **Arch. Virol**. 141: 331-344.

Verdaguer, N., M.G. Mateu, D. Andreu, E.Giralt, E. Domingo and I. Fita. 1995. Structure of the major antigenic loop of foot and mouth disease virus complexed with a neutralizing antibody: direct involvement of the Arg-Gly-Asp motif in the interaction. **EMBO J.** 14: 1690-1696.

_____, G. Schoehn, W.F. Ochoa, I. Fita, S. Brookes, A. King, E. Domingo, M.G. Mateu, D. Stuart and E.A. Hewatt. 1999. Flexibility of the major antigenic loop of foot-and-mouth disease virus bound to Fab fragment of neutralizing antibody: structure and neutralization. **Virology.** 255: 260-268.

_____, N. Sevilla, M. Valero, D. Stuart, E. Brocchi, D. Andreu, E. Giralt, E. Domingo, M.G. Mateu and I. Fita. 1998. A similar pattern of interaction for different antibodies with a major antigenic site of foot and mouth disease virus implications for intertypic antigenic variation. **J. Virol**. 72: 739-748.

Volpina, O.M., A. Y. Surovoy, M.N. Zhmak, M.A. Kuprianova, D.O. Koroev, A.V. Chepurkin, A.S. Toloknov and V.T. Ivanov. 1999. A peptide construct containing B cell and T-cell epitopes from the foot and mouth disease viral VP1 protein induces efficient antiviral protection. **Vaccine**. 17: 577-584.

_____, A.V. Yarov, M.N. Zhmak, M.A. Kuprianova, A.V. Chepurkin, A.S. Toloknov and V.T. Ivanov. 1996. Synthetic vaccine against foot and mouth disease based on a palmitoyl derivative of the VP1 protein 135-159 fragment of the A22 virus strain. **Vaccine**. 14: 1375-1380.

Wajjwalku, W., S. Kasemsuwan, W. Chumsing, J. Wongsanit, A. Yawongsa, K. Tancharoen, N. Tongthip, W. Rungrattanaubol and T. Pinyopummintr. 1999. Isolation of foot and mouth disease virus from clinical cases. pp. 388-391. *In* **The 37th Kasetsart University Annual Conference**, Kasetsart University, Bangkok.

Wang, C.Y., T.Y. Chang, A.M. Walfield, J. Ye, M. Shen, S.P. Chen, M.C. Li, Y.L. Lin, M.H. Jong, P.C. Yang, N. Chyr, E. Kramer and F. Brown. 2002. Effective synthetic peptide vaccine for foot and mouth disease in swine. **Vaccine**. 3175:1-8.

_____, _____, _____, _____, _____, M.L. Zhang, J. Lubroth, S.P. Chen, M.C. Li, Y.L. Lin, M.H. Jong, P.C. Yang, N. Chyr, E. Kramer and F. Brown. 2001. Synthetic peptide base vaccine and diagnostic system for effective control of FMD. **Biologicals**. 29: 221-228.

Wang, J.H., C.M. Liang, J.M. Peng, J.J. Shieh, M.H. Jong, Y.L. Lin, M. Sieber and S.M. Liang. 2003. Induction of immunity in swine by purified recombinant VP1 of foot and mouth disease virus. **Vaccine.** 21: 3721-3729.

Wigdorovitz, A., C. Carrillo, M.J. Dus Santos, K. Trono, A. Peralta, M.C. Gomez, R.D. Rios, P.M. Franzone, A.M. Sadir, J.M. Escribano and M.V. Borca. 1999. Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. **Virology.** 255: 347-353.

Wong, H.T., S.C.S. Cheng, E.W.C. Chan, Z.T. Sheng, W.Y. Yan, Z.X. Zheng and Y. Xie. 2000. Plasmids encoding foot and mouth disease virus VP1 epitopes elicited immune responses in mice and swine and protected swine against viral infection. **Virology.** 278: 27-35.

_____, _____, F.W.Y. Sin, E.W.C. Chan, Z.T. Sheng, and Y. Xie. 2002. A DNA vaccine against foot and mouth disease elitist an immune response in swine which is enhanced by co-administration with interleukin-2. **Vaccine.** 20: 2641-2647.

Zamorano, P., A. Wigdorovitz, M. Perez-Filgueira, C. Canrrillo, J.M. Escribano, A.M. Sadir and M.V. Borca. 1995. A 10-amino-acid linear sequence of VP1 of foot and mouth disease virus containing B and T cell epitopes induces protection in mice. **Virology.** 212: 614-621.

Zhang, Z. and P. Kitching. 2000. A sensitive method for the detection of foot and mouth disease by in situ hybridisation using biotin-labelled oligodeoxynucleotides and tyramide signal amplification. **J. Virol. Methods**. 88: 187-192.

Zhang, H.Y.,S.H. Sun, Y.J. Guo, F.J. Zhou, Z.H. Chen, Y. Lin and K. Shi. 2003. Immune response in mice inoculated with plasmid DNAs containing multiple-epitopes of foot and mouth disease virus. **Vaccine**. 21:4704-4707.

APPENDIX

APPENDIX A

Chemical Reagents and Substances

1. Cocktail of RobusT I RT-PCR KIT (FINNZYMES)

5 μl 10X Robus T reaction buffer

1.5 μl 50mM $MgCl_2$ solution

1 μl dNTP mix (10mM each)

1 μl AMV Reverse Transcriptase

optimal concentration of RNA Template

100 pmol Hexamer

Add RNAse free water to 50 μl

2. Cocktail of Dynazyme EXT kit (FINNZYMES)

10 μl 10X Dynazyme EXT reaction buffer

2 μl dNTP mix (10mM each)

3 μl 50mM $MgCl_2$ solution

100 pmol Forward primer

100 pmol Reverse primer

Optimal concentration of cDNA Template

0.5 μl DyNAzyme EXT DNA polymerase

Add Sterile water to 100100 pmol

3. Reagent for RNA Extraction

- D solution

 4M guanidine thiocyanate

 25mM Sodium citrate, pH7.0

 0.5% Sarcosyl

3. Buffer for agarose gel electrophoresis

- 20X TAE buffer pH 8.3 stock solution

 0.8 M Tris HCl

 0.4 M sodium acetate

 0.04 M Na_2EDTA

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

- 10X loading buffer / dye

 20% glycerol

 0.01% bromphenol blue

 add TE to final volume

- 5 mg/ml ethidium bromide (EtBr)

 500 mg EtBr

 add distilled water to 100 ml

4. Bacterial media and solution

- Luria Bertani (LB) medium (broth)

 10 g tryptone

 5 g yeast extract

 10 g NaCl

Distill water add up to 1liter

- Ampicillin stock solution

  100 mg/ml in H_2O, sterile filter, store in aliquots at –20°C

- IPTG (1 M)

  238 mg/ml in H_2O, sterile filter, store in aliquots at –20°C

5. Reagent for ELISA

- Coating Buffer pH9.6 (5X concentrated)

  1.12 g Na_2CO_3

  2.94 g $NaHCO_3$

  0.20 g NaN_3

  Distill water add up to 200 ml

  Adjust pH to 9.6

- Phosphate buffered saline (PBS)

  0.2 g KH2PO4

  2.9 g Na2HPO2 12H2O

  8.0 g NaCl

  0.2 g KCL

  Distill water add up to 200ml

- Blocking buffer

  400g Sucrose

  5 ml Triton X-100

  20g Casein

  Distill water add up to10liters

  Adjust pH to 7.4

6. Media for myeloma and hybridoma

- Culture medium (CM)

  1 pack HyQ RPMI 1640 medium (HyClon®)

  2 g $NaHCO_2$

  1X MEM Non Essential Amino Acid 100X (PAA®)

  100U/ml Penicillin

  100 mg/ml Streptomycin

  Add distilled water to 1liter, sterilize by filter and store at 4°C

- HT medium

  CM medium

  10%Fetal Bovine Serum (HyClon®)

  1X HT Supplement 100X (Gibco™)

- HAT medium

  CM medium

  10% Fetal Bovine Serum (HyClon®)

  1X HAT Supplement 50X (Gibco™)

7. SDS-PAGE reagents

- 2x SDS-PAGE sample buffer

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

  2.0 ml of glycerol (20% glycerol)

  0.4 g of SDS or 4 ml of 10%SDS (4% SDS)

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

  0.006% bromophenol blue

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

  0.5M TrisCl

  0.4% SDS

  Distilled water 200 ml

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

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

  1.5M TrisCl

  0.4% SDS

  Distilled water 400 ml

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

- 30% Acrylamide / Bis-acrylamide (bis-acrylamide : acrylamide = 1:36)

  To prepare 513.5 ml of solution ;

  Acrylamide 150 g

  Bis-acrylamide 4.1 g

  Add distilled water to 513.5 ml, sterilize by filter and store at $4^{o}C$

- 10% Ammoniun persulfate

  100 mg Ammonium persulfate

  1 ml distilled water

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

  Store protected from light at $4^{o}C$

- Glycine buffer

  192 mM glycine

  25 mM Tris base

  0.1% SDS

- Coomasie Brilliant Blue stain (2 litres)

  2 g Coomasie brilliant blue powder

  1 litre methanol

  200 ml acetic acid

  800 ml distilled water

  Stir for minimun 2 hours and filter through Whatman filter disc

- Destainning solution (100 ml)

  225 ml methanol

10 ml acetic acid

225 ml distilled water

8. Copper staining reagents

- Staining solution

  0.3 M $CuCl_2$

- Destainning solution

  0.25 M EDTA

  0.25 M Tris (pH9.0)

9. DAB substrate solution

- Staining solution

  6mM DAB (3,3'-diaminobenzidine)

  10 ml Tris buffer pH7.8

  100 µl 3% H_2O_2

APPENDIX B

The standard methods

1. **Agarose gel electrophoresis (Sambrook *et al.*, 1989)**

1.1 Prepare an agarose gel, according to recipes list e.g. 1% gel in 1XTAE buffer, by combining the agarose with buffer in the Erlenmeyer flask and wrapped with clear pored plastic (low gel temperature agarose may also be used) and melt the agarose for 3 minutes by microwave oven. Check the gel temperature before pouring the gel onto plate, the desired temperature is about 55-60 $^{\circ}$C.

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

1.3 Carefully remove the tape and the gel casting combs and place the gel

in a horizontal electrophoresis apparatus. Add 1x TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.

1.4 Add at least one- tenth volume of 5x 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.

1.5 Incubate the agarose gel in EtBr tank for 15-20 minutes.

1.6 Visualize the DNA fragments on a long wave UV light box.

2. **SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook *et al.*, 1989)**

Prepare the gel by the recipe as follow for 2 gels

- Separating gel (12%)

H_2O	2.8 ml

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

- Stacking gel

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

If not already in electrophoresis sample buffer, add an equal volume of 2X sample buffer to all samples and boil for 5 minutes. Apply 20-25 µl (1 – 10 µg total protein) of cell lysate to each well of a 0.75-1.0 mm thick gel. Run electrophoresis (100 volt, 70 mins, constant ampere)

3. <u>Copper staining</u>

3.1 Wash gel with distilled eater

3.2 Place the gel in glass or plastic tray

3.3 Add at least 5 volumes of staining solution

3.4 Incubate at room temperature with agitation for 5 minutes

3.5 Wash the gel with destaining solution

3.6 Observe against a dark background

APPENDIX C

Manufacturer's instruction of commercial test kit

1. UBI® FMDV NS EIA (Swine) and UBI® FMDV VP1 EIA (Swine)

1.1 Assay procedure

1.1.1 Separate DILUTION MICROPLATES are provided for specimen dilution.

- Use two wells (A1 and B1) for FMDV NON-REACTIVE CONTROL.
- Use two wells (C1 and D1) for FMDV NS REACTIVE CONTROL.
- Use one or two wells for each specimen.
- Place the DILUTION MICROPLATES over the MICROWELL LOCATOR LABEL (optional)
- Dilute the Controls and Specimens as follows (1:21)
- Add 200 μl of SPECIMEN DILUENT into each CONTRL or SPECIMEN well of the DILUENT MICROPLATE
- Dispense 10 μl of Controls or Specimens into the assigned wells of the DILUTION MICROPLATE. Mix thoroughly by pipetting up and down 3 times. Use a separate pipette tip for each diluted CONTROL or SPECIMEN.

1.1.2 Cut open the foil pouch and remove the REACTION MICROPLATE. If necessary, remove any excess antigen-coated strips from the frame and return them to the foil pouch. Seal the extra strips securely inside the foil pouch making sure to include the desiccant provided. Return to storage at 2 C to 8 C immediately. It may be necessary to replace these strips with blank strips (not provided in the kit), depending on the washing system used.

1.1.3 Transfer 100 μl of the diluted Controls and Specimens from each well of the DILUTION MICROPLATE to its corresponding well in the REACTION MICROPLATE. Dispose of the DILUTION MICROPLATE and unused liquid as biohazardous waste.

1.1.4 Within 30 minutes of SPECIMEN/CONTROL addition, cover with the enclosed lid or equivalent and incubate for 60± 2C

1.1.5 Dilute the CONJUGATE 1:101 with CONJUGATE DILUENT prior to washing the microplates (WORKING CONJUGATE).

1.1.6 Uncover the REACTION MICROPLATE and wash with diluted WASH BUFFER.

1.1.7 After washing is complete, excess liquid may be removed from the REACTION MICROPLATE by inverting and rapping on the absorbent paper until no further moisture appears on the paper.

1.1.8 Add 100 μl of the WORKING CONJUGATE solution (1:101) to all wells of the REACTIOM MICROPLATE

1.1.9 Cover and incubate for 30± 2C

1.1.10 Uncover and repeat the wash procedure as in steps 6 and 7

1.1.11 Add 100 μl of TMB SUBSTRATE SOLUTON to each well of the REACTION MICROPLATE.

1.1.12 Cover and incubate in the dark for 15± minutes at 37 ± 2C

1.1.13 Uncover and add 100 μl of STOP SOLUTION to each well of the REACTION MICROPKATE. Mix by gently tapping the side of the reaction.

1.1.14 Read the absorbance of well at 45o nm. Blank on air.

1.1.15 Review absorbance values for the Controls. Refer to CALCULATION OF RESULTS for control validation parameters and calculation procedures.

1.2 Calculation of results

1.2.1 Determine the mean of the FMDV NON-REACTIVE CONTROLs (NRC) and FMDV NS REACTION CONTROLs (RC)

1.2.2 Calculation of the CUTOFF VALUE

CUTOFF VALUE = (0.23) X (RC)

1.3 Interpretation of results

1.3.1 Specimens with absorbance values less than the CUTOFF VALUE are considered non-reactive by the criteria of the UBI® FMDV NONSTRUCTURAK PROTEIN ELISA

1.3.2 Specimens with absorbance values greater than or equal to the CUTOFF VALUE are considered initially reactive

2. Ceditest® FMDV-NS

2.1 Test procedure

Day1; Incubation with test serum

- Dispense 80 μl ELISA buffer to all wells.
- Dispense 20 μl of negative control to wells A1 and B1
- Dispense 20 μl of weak positive control to wells C1 and D1.
- Dispense 20 μl of positive control to well E1 and F1.
- Dispense 20 μl of test samples to remaining wells
- Seal the test plate(s) using the enclosed plate sealers.
- Shank the test plate(s) gently.
- Incubate overnight (16-18 hrs) at room temperature (20-25C)

Day2 ; Incubation with conjugate and chromogen/substrate solution

- Empty the test plate(s) after the incubation period and wash the plate(s) 6 times with washing fluid. Tap the plate(s) firmly after the late washing.
- Dispense 100 μl of the working dilution of the conjugate to all wells.
- Seal the test plate(s) using the enclosed plate sealers
- Incubate 1 hour at room temperature (20-25C).
- Empty the test plate(s) after the incubation period and wash the plate(s) 6 times with washing fluid. Tap the plate(s) firmly after the late washing.
- Dispense 100 μl of chromogen/substrate solution to all wells.
- Incubate 20 minutes at room temperature (20-25C).
- Add 100 μl of the stop solution to all wells.
- Mix the content of the wells of the test plate(s) prior to measuring.

2.2 Reading of the test plate(s) and calculating the results

- Measure the optical density (OD) of the wells at 450 nm preferable within 15 minutes after color development has been stopped.

- Calculate the mean OD_{450} value of wells A1 and B1 (negative control=OD max).

- the percentage inhibition (PI) of the controls and the test sera are calculated according to the formula below

$$PI = 100 - \left(\frac{\underline{OD450test\ sample}}{OD450max} \times 100\right)$$

2.3 Interpretation of the percentage inhibition

PI= < 50% Negative   no antibodies against the NS protein of FMDV

PI= > 50% Positive   antibodies against the NS protein of FMDV

3. <u>FMD-3ABC po (CHEKiT®)</u>

3.1 Procedure

3.1.1 Allow all reagents to equilibrate to the required incubation temperature.

3.1.2 if only a portion of a microtiter stripped plate id required, it is possible to cut the foil sealing the plate and remove the strips which are not needed for the assay.

3.1.3 Determine the amount of CHEKIT- Washing&Dilution-section needed fot washing the microtiter plates, and diluting conjugate. Dilute the CHEKIT- 10X-Concentrate 1:10 with water (1 part concentrate with 9 parts water).

3.1.4 Dispense 90 μl CHIKIT-FMD 3ABC-sample-Diluent into each well of the the microtiter plate.

3.1.5 Add 10 μl of the undiluted samples and controls into the appropriate wells of the microtiter plate. Final dilution =1:10.

- Use two wells (A1 and A2) for Negative CONTROL.
- Use two wells (B1 and B2) for Positive CONTROL.
- Use one or two wells for each specimen.

3.1.6 Mix the contents within each well by gently shaking the microtiter plate briefly.

3.1.7 Cover the microtiter plate with a lid and incubate for 60 minutes at 37 C ± 2C in a humid chamber.

3.1.8 After the incubation, wash 3 times each microtiter plate.

3.1.9 the CHEKIT-Anti-IgG-Po-Conjugate 1:200 by using CHEKIT-Washing&Dilution-Solution

3.1.10 100 μl of this dilution into each well, cover and incubate the covered microtiter plate for 60 minutes at 37C ± 2C in a humid chamber.

3.1.11 Wash 3 times

3.1.12 Dispense 100 μl CHEKIT-Chromogen, pre-warmed to 25 C into each well.

3.1.13 Read the results with a photometer at a wavelength of 405 nm (reference wavelength =492nm). As soon as the difference in the OD between the negative and positive controls is $\geq$ 0.4, the reaction can be stopped by adding 50 ul per well of CHEKIT-Stopping-Solution, prewarmed to room temperature.

3.2 Interpretation of results

The OD of the positive control (ODpos) as well as the OD of the samples (ODsample) are corrected by subtracting the OD of the negative control (ODneg).

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

$$\text{Value}(\%) = \frac{\text{ODsample-ODneg}}{\text{ODpos-ODneg}} \text{X100}$$

Value < 20% = negative

Value 20-30% = ambiguous

Value >30% = positive

4. Mouse MonoAb ID KIT (HRP) (ZYMED®)

4.1 Procedure

4.1.1 Add 50 μl of optimal antigen dilution in all wells of plate and incubate at 4°C overnight.

4.1.2 Decants and slap onto until dry.

4.1.3 Add 1% BSA to all well and incubate at 37°C for 1hour.

4.1.4 Decant and slap onto a paper towel

4.1.5 Add 50 μl first sample each well in the first row and one additional sample add to each subsequent row.

4.1.6 Incubate at 37°C for 30minutes and wash 4 times with PBS-Tween.

4.1.7 Add the buffer in the first column is blank. Second column for negative control add 1 drop of normal rabbit serum to each well and another columns add 1 drop of each subclass specific antibodies.

4.1.8 Incubate at 37°C for 30minutes and wash 4 times with PBS-Tween.

4.1.9 Add 50ul of diluted HRP-Goat Anti-Rabbit IgG (H+L) to all wells and incubate at 37°C for 30 minutes.

4.1.10 wash 4 times with PBS-Tween before add 100 μl of working substrate solution to all wells and incubate at room temperature for 30 minutes.

4.1.11 read quantitatively with a spectrophotometer at 405 nm.

APPENDIX D

Nucleotide Sequencing of 3AB and Nomenclature of the 20 Standard Amino Acids

```
             10        20        30        40        50        60
     ....|....|....|....|....|....|....|....|....|....|....|....|
1    TTCAAGCAGATCTCGATTCCTTCTCAAAAGTCTGTGCTGTACTTCCTCATTGAGAAAGGC 60
      F  K  Q  I  S  I  P  S  Q  K  S  V  L  Y  F  L  I  E  K  G

             70        80        90       100       110       120
     ....|....|....|....|....|....|....|....|....|....|....|....|
61   CAGCACGAAGCAGCAATTGAATTCTTTGAGGGGATGGTGCATGACTCCATTAAGGAGGAG 120
      Q  H  E  A  A  I  E  F  F  E  G  M  V  H  D  S  I  K  E  E

            130       140       150       160       170       180
     ....|....|....|....|....|....|....|....|....|....|....|....|
121  CTCCGGCCCCTCATCCAACAAACCTCATTTGTGAAACGCGCTTTTAAGCGCCTGAAGGAA 180
      L  R  P  L  I  Q  Q  T  S  F  V  K  R  A  F  K  R  L  K  E

            190       200       210       220       230       240
     ....|....|....|....|....|....|....|....|....|....|....|....|
181  AACTTTGAGATTGTTGCCCTATGTTTGACTCTCCTGGCAAACATAGTGATCATGATCCGC 240
      N  F  E  I  V  A  L  C  L  T  L  L  A  N  I  V  I  M  I  R

            250       260       270       280       290       300
     ....|....|....|....|....|....|....|....|....|....|....|....|
241  GAGACTCGTAAGAGGAAGCAAATGGTGGATGATGCAGTGAATGAGTACATTGAGAAGGCA 300
      E  T  R  K  R  K  Q  M  V  D  D  A  V  N  E  Y  I  E  K  A

            310       320       330       340       350       360
     ....|....|....|....|....|....|....|....|....|....|....|....|
301  AACATCACCACAGACGACAAAACTCTTGACGAGGCGGAAAAGAACCCTCTGGAGACCAGT 360
      N  I  T  T  D  D  K  T  L  D  E  A  E  K  N  P  L  E  T  S

            370       380       390       400       410       420
     ....|....|....|....|....|....|....|....|....|....|....|....|
361  GGCGCCAGTACCGTTGGCTTCAGAGAGAGATCTCTCCCCGGACACAAGGTGAGTGATGAC 420
      G  A  S  T  V  G  F  R  E  R  S  L  P  G  H  K  V  S  D  D

            430       440       450       460       470       480
     ....|....|....|....|....|....|....|....|....|....|....|....|
421  GTGAACTCCGAGCCCACCAAGCCCGCGGAAGAGCAACCACAAGCTGAAGGACCCTACGCC 480
      V  N  S  E  P  T  K  P  A  E  E  Q  P  Q  A  E  G  P  Y  A

            490       500       510       520       530       540
     ....|....|....|....|....|....|....|....|....|....|....|....|
481  GGACCACTCGAGCGTCAGAAACCTCTGAAAGTGAAAGCCAAGCTGCCACAGCAGGAGGGA 540
      G  P  L  E  R  Q  K  P  L  K  V  K  A  K  L  P  Q  Q  E  G

            550       560       570       580       590       600
     ....|....|....|....|....|....|....|....|....|....|....|....|
541  CCCTACGCTGGTCCGATGGAGAGACAGAAACCACTGAAAGTGAAAGCAAAAGCCCCGGTC 600
      P  Y  A  G  P  M  E  R  Q  K  P  L  K  V  K  A  K  A  P  V

            610       620       630       640       650       660
     ....|....|....|....|....|....|....|....|....|....|....|....|
601  GTGAAGGAAGGACCTTACGAAGGACCGGTGAAGAAGCCTGTCGCTTTGAAAGTGAAAGCT 660
      V  K  E  G  P  Y  E  G  P  V  K  K  P  V  A  L  K  V  K  A

            670       680       690
     ....|....|....|....|....|....|.
661  AAGAACTTGATTGTCACTGAGAGTGGATCCC 691
      K  N  L  I  V  T  E  S  G  S
```

Appendix Figure D1 Nucleotide sequencing of 3AB PCR product using cloning and expressed in *E.colli.*

Source: Letwatcharasarakul (2000)

Appendix Table D1 Nomenclature of the 20 standard amino acids

Amino Acid	Three-Letter Symbol	One-Letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Th reonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX E

The r-Values of Serotype O from Liquid Phase Blocking ELISA

Appendix Table E1 the r-values of serotype O from liquid phase blocking ELISA were obtained from the Regional Reference Laboratory for FMD in South East Asia (RRL).

Virus Designation	Genotype/Lineage	r-value
O/35/PCBR/00	IA	0.38
O/02/MHS/99	IA	1
O/19/CR/00	IA	1
O/42/NAN/00	IA	0.44
O/54/CM/01	IB	1
O/26/YL/00	IIA	0.75
O/49/NRTW/00	IIA	0.94
O/30/SK/00	IIA	1
O/25/PTN/00	IIA	1
O/53/CR/01	IIC	0.5
O/48/ANCR/01	IID	1
O/44/BRR/99	IIIA	1
O/16/ANCR/00	IIIB	0.63
O/38/CM/00	IIIC	0.5
O/15/SKNK/00	Pan Asia	0.43
O/9/CR/99	Pan Asia	0.67
O/8/CR/99	Pan Asia	1
O/14/NK/00	Pan Asia	0.5
O/5/YST/99	Pan Asia	0.5
O/18/UB/00	Pan Asia	0.67
O/32/UB/00	Pan Asia	0.5
O/24/UB/00	Pan Asia	0.69
0/52/CM/01	Pan Asia	0.67
O/22/CR/00	Pan Asia	1

CURRICULUM VITAE

NAME	Mr.Preeda Lertwatcharasarakul		
BIRTH DATE	October 23, 1976		
BIRTH PLACE	Bangkok, Thailand		
EDUCATION	YEAR	INSTITUTION	DEGREE/DIPLOMA
	1998	Mahidol Univ.	Bachelor's Degree
	2000	Kasetsart Univ.	Master's Degree
POSITION/TITLE	-		
WORK PLACE	-		
SCHOLARSHIP/AWARDS	University Staff Academic Development Project, under The Ministry of University Affairs		