Application of needle-free injection technology for foot and mouth disease vaccination via intradermal route

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โรคปากและเท้าเปื่อยเป็นโรคประจำถิ่นในประเทศไทย ก่อให้เกิดความสูญเสียทางเศรษฐกิจ การป้องกันและควบคุมโรคปากและเท้าเปื่อยสามารถปฏิบัติโดยการให้วัคซีนโดยการฉีดเข้าใต้ผิวหนังแก่สัตว์ปีละ 2-3 ครั้ง ถึงแม้ว่ามีการฉีดวัคซีนอย่างต่อเนื่องยังคงพบการระบาดของโรคปากและเท้าเปื่อย สาเหตุของการระบาดอาจเกิดจากการเพิ่มระดับภูมิคุ้มกันที่ไม่เพียงพอในประชากรสัตว์ การกระจายวัคซีนไม่ทั่วถึง และเกษตรกรไม่สามารถจับบังคับสัตว์ทำให้ไม่สามารถให้วัคซีนแก่สัตว์ได้ จากหลายการศึกษาพบว่า วิธีการบริหารยาโดยการฉีดวัคซีนเข้าในผิวหนังสามารถกระตุ้นภูมิคุ้มกันได้ด้วยปริมาณที่ต่ำกว่าการฉีดเข้าใต้ผิวหนังและการประยุกต์ใช้วัคซีนโดยการฉีดเข้าผิวหนังด้วยเทคโนโลยีการฉีดยาที่ปราศจากเข็มจะเป็นทางเลือกที่มีศักยภาพในการทำวัคซีนในโคนม ทำให้สามารถมีจำนวนวัคซีนมากขึ้น และการฉีดยาอัตโนมัติแบบปราศจากเข็มโดยการฉีดเข้าในผิวหนังซึ่งใช้งานง่าย สะดวก มีระบบการทำงานที่มีประสิทธิภาพ สามารถทำวัคซีนแก่สัตว์ได้สะดวก ดังนั้นวัตถุประสงค์ของการศึกษา เพื่อเปรียบเทียบระดับภูมิคุ้มกันจากการทำวัคซีนโรคปากและเท้าเปื่อยด้วยวิธีการฉีดวัคซีนและศึกษาปริมาณโด้สในบริหารการฉีดเข้าในผิวหนังที่สามารถกระตุ้นระดับภูมิคุ้มกันจากวัคซีน แบ่งการศึกษาออกเป็น ในลูกโค 40 ตัว และโคสาว 40 ตัว การศึกษาในลูกโค แบ่งออกเป็น เจ็ดกลุ่ม กลุ่มละ 5 ตัวยกเว้นกลุ่มที่สอง 10 ตัว กลุ่มที่หนึ่ง ได้รับน้ำเกลือเข้าในผิวหนังปริมาณ 1 มิลลิลิตร กลุ่มที่สอง ได้รับวัคซีนเข้าใต้ผิวหนังปริมาณ 2 มิลลิลิตร กลุ่มที่สาม ได้รับวัคซีนเข้าผิวหนังด้วยเทคโนโลยีที่ปราศจากเข็มปริมาณ 1 มิลลิลิตร กลุ่มที่สี่ถึงเจ็ดได้รับวัคซีนเข้าผิวหนังปริมาณ 0.25, 0.5, 1 และ 2 มิลลิลิตร ตามลำดับ การศึกษาในโคสาวแบ่งออกเป็น สี่กลุ่มกลุ่มละ 10 ตัว กลุ่มที่หนึ่ง ได้รับวัคซีนเข้าใต้ผิวหนังปริมาณ 2 มิลลิลิตร กลุ่มที่สอง ได้รับวัคซีนเข้าใต้ผิวหนังโดยเทคโนโลยีปราศจากเข็ม ปริมาณ 2 มิลลิลิตร กลุ่มที่สาม ได้รับวัคซีนเข้าในผิวหนังปริมาณ 1 มิลลิลิตร และกลุ่มที่สี่ ได้รับวัคซีนเข้าในผิวหนังด้วยเทคโนโลยีที่ปราศจากเข็มปริมาณ 1 มิลลิลิตร ลูกโคได้รับวัคซีนเข็มแรกในวันแรกของการศึกษาและกระตุ้นซ้ำในวันที่ 14 ของการศึกษา ส่วนโคสาวได้รับการกระตุ้นวัคซีน 1 ครั้งในวันแรกของการศึกษา สัตว์ในการศึกษาจะถูกเก็บเลือดตั้งแต่วันแรกของการศึกษาจนถึงวันที่หนึ่งร้อยยี่สิบ เพื่อประเมินระดับภูมิคุ้มกันหลังจากการทำวัคซีนโดยวิธีการตรวจแบบไวรัสนิวเทลไรซิ่งและการตรวจโปรตีนเอ็นเอสพีเพื่อประเมินการติดเชื้อโดยธรรมชาติ ผลการศึกษาพบว่าไม่พบระดับแอนติบอดี้ต่อโปรตีนเอ็นเอสพีใน 98 ตัวอย่างจากกลุ่มลูกโค 25 ตัวที่ตรวจ ระดับนิวเทลไรซิ่งแอนติบอดีต่อซีโรไทป์โอในลูกโค ในวันที่ 7 หลังจากการให้วัคซีนพบว่า กลุ่มหก มีค่าสูงสุดตามด้วย กลุ่มสี่ ในขณะที่วันที่ 21 ของการศึกษาพบว่ากลุ่มสี่มีค่าสูงสุดตามด้วยกลุ่มหก เมื่อเทียบสัดส่วนของสัตว์ที่มีระดับภูมิคุ้มกันถึงระดับป้องกันโรคได้ พบว่าลูกโคมีระดับภูมิคุ้มโรคต่อซีโรไทป์โอมีค่าประมาน 40% อย่างไรก็ตาม ผลการศึกษาของโคสาวพบว่า สัดส่วนของสัตว์ที่มีระดับภูมิคุ้มโรคต่อซีโรไทป์โอในวันที่ 7 ของการศึกษามีค่าประมาณ 80% โดยไม่พบความแตกต่างระหว่างกลุ่ม ผลการศึกษานี้สามารถบ่งชี้ ระดับนิวเทลไรซิ่งแอนติบอดีในลูกโคหลังจากการทำวัคซีนมีค่าต่ำ ทั้งในกลุ่มที่ใช้เทคโนโลยีการฉีดยาแบบปราศจากเข็มและการฉีดเข้าใต้ผิวหนังโดยไม่แตกต่างกัน วัคซีนโรคปากและเท้าเปื่อยเป็นวัคซีนเชื้อตายซึ่งกระตุ้นภูมิคุ้มกันโรคได้ต่ำ ระยะคุ้มกันโรคสั้น ดังนั้นการทำวัคซีนในลูกโคจึงจำเป็นต้องมีการกระตุ้นซ้ำหลายๆครั้ง ผลการศึกษาในโคสาวพบว่า การให้วัคซีนในผิวหนังด้วยเทคโนโลยีที่ปราศจากเข็มปริมาน 1 มิลลิลิตรสามารถใช้ทดแทนวิธีการให้วัคซีนใต้ผิวหนังปริมาณ 2 มิลลิลิตรได้ และสามารถลดขนาดโด๊สของวัคซีนลง ดังนั้นการฉีดยาด้วยเทคโนโลยีที่ปราศจากเข็มทางผิวหนังสามารถนำมาเป็นทางเลือกของการฉีดวัคซีนป้องกันโรคปากและเท้าเปื่อยในโคนมของประเทศไทย

สาขาวิชา	อายุรศาสตร์สัตวแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: Dairy cattle, foot-and-mouth disease, intradermal vaccination, needle-free device, subcutaneous vaccination

Sirirat Wataradee : Application of needle-free injection technology for foot and mouth disease vaccination via intradermal route. Advisor: Asst. Prof. Dr. CHAIDATE INCHAISRI, D.V.M., M.Sc., Ph.D., D.T.B.V.M. Co-advisor: Assoc. Prof. Dr. KITTISAK AJARIYAKHAJORN, D.V.M., M.Sc., Ph.D.

Foot and mouth disease (FMD) is an endemic disease in Thailand and caused severe economic loses. In order to prevent and control disease, the vaccination program is routinely administered for 2-3 times a year via subcutaneous route (SC). Even though, the vaccination is performed regularly, the outbreaks have often reported. The outbreaks occur due to the failure of number of immunity animal in population or herd immunity. The limitation of vaccine distribution to all animal population and difficulty of cattle restraint are forcible several farmers to failing to vaccinate their animals. Based on previous studies, the intradermal route (ID) can induce an efficient immune response with a lower dose than the SC route. Recently, the automatic needle-free intradermal vaccination has been applied as an alternative vaccinating method in dairy cows. The automatic needle-free intradermal vaccination offers the rapid and practical vaccine administration. Therefore, this study aims to compare the immune response between different routes of FMD vaccine administration and to optimize dose of ID vaccine. The conducted study used 40 calves and 40 heifers with inactivated FMD trivalent (O, A and Asia-1) vaccine in which produced locally by department of livestock development (DLD). The calves were allocated into seven groups of five calves per group (except 10 in group II) and vaccinated as group I: ID injected placebo (1 mL normal saline), group II: SC vaccinated with 2 mL, group III: ID vaccinated via automatic needle-free device with 1 mL, groups IV-VII: ID vaccinated with 0.25 mL, 0.5 mL, 1 mL and 2 mL, respectively. Additionally, heifers were divided into four group of ten animals each. Group I 2 mL SC vaccination, group II 2 mL SC vaccination via automatic needle-free device with 2 mL, group III 1 mL vaccination and group IV 1 mL ID vaccination via automatic needle-free device. Calves were vaccinated twice (day 0 and day 14), while heifers were vaccinated only once with trivalent FMDV vaccine. Blood samples were collected from 0 to 120 days post-vaccination (dpv) to determine the immune response by viral neutralization test (VNT). To check status of FMD infection in experimental animal, the level of antibody against non-structural protein of virus (NSP) was measured by PrioCHECK FMDV NS ELISA tests. The result found that the 98 selected samples from 25 calves were found sero-negative for NSP antibodies. The highest average NAT against serotype O on 7 dpv was group VI (ID 1) followed by group IV (ID 0.25), while the highest average against serotype Asia-1 NAT on 7 dpv was group IV (ID 0.25) followed by group VI (ID 1). The levels of a proportion of protective levels against serotype O in calves was mostly lower than 40%. However, the result of the heifers found that the highest average NAT against serotype O on 7 dpv was mostly higher than 80% of the proportion of protective levels with no significant among groups. The results reveal that mostly NAT values in calves were low in which the NAT values from ID via automatic needle-free (group III) did not differ from SC (group II). Since the inactivated FMD vaccine has stimulated the low immune response with a short duration in protection, therefore, the vaccination in calves should be intense boosted. The results in heifers illustrated that automatic needle-free device via ID 1 mL can be a substitute for the SC. In order to reduce dosage of vaccine, the application of ID via an automatic needle-free device can be considered as the alternative for FMD vaccination in Thai dairy cattle.

Field of Study:	Veterinary Medicine	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

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Chapter 1: Introduction

Foot-and-mount disease (FMD) is an endemic disease in Thailand. FMD is a highly contagious disease in cloven-hoofed animals such as cattle, small ruminant and swine. The disease causes severe production losses in which the majority of recovery affected animals become weakened and debilitated. Economic loss caused by FMD which represented as: direct loss is impaired production, infertility and mortality whereas, indirect loss is from the cost of prevention and control.

Regarding FMD prevention strategy issued by department of livestock development (DLD) cattle must be vaccinated two to three times per year. Even though vaccination is conducted routinely, many outbreaks still occurred. The DLD provides trivalent FMD vaccine (against the FMD virus (FMDV) serotype A, O and Asia-1) for all ruminant population. The recommendation for FMD vaccination is injected with 2 mL per dose FMD subcutaneous route (SC) in ruminant.

According to the demand and supply of FMD vaccine in Thailand, about 14-21 million doses FMD vaccine is required in which calculation is based on a number of ruminant populations (Office of Agricultural Economics, 2016). Without a good vaccine management, FMD vaccine seems being insufficient to increase the protective immunity for all animals in a country. As shown in the report of FMD serological survey of DLD in 2006 found that the immunity to protect FMD at the herd level was less than 60% of population and the percentage of beef cattle with protective immunity against FMDV serotype O and A at the animal level was about 50% of beef cattle population (Jithlang and Sirimongkolrat, 2008). However, low level of protective of protection may be caused either by the failure to vaccinate or by the actual vaccine failure. The vaccination failures may be due to vaccine

failure including vaccine strain mismatched with viral field strain, poor quality vaccine and failure of cold chain management. Vaccine is improper injected, farmer refused to vaccinate animal due to the difficulty animal restraint and the insufficiency of vaccine quantity (Pay, 1985; Doel, 1996; Keeling et al., 2003; Elnekave et al., 2016b).

Several studies have shown that intradermal (ID) vaccination is alternative route of immunization. ID layer is particularly relevant with many of dendritic cells which is potent antigen-presenting cells (APCs) to induce protective immunity (Hunsaker and Perino, 2001). ID vaccination route is generally able to stimulate faster immune response than SC route (Hunsaker and Perino, 2001; Eble et al., 2009). In addition, comparison with conventional route, ID route reduces the amount of vaccine administered per dose. For example, ID vaccination of new licensed influenza and rabies vaccine has been shown to induce immune response equivalent to the standard dose of conventional route (Hickling and Jones, 2009). Several studies have shown that the ID vaccination with less amount of vaccine can stimulate an immune response, in addition, ID route can rapidly stimulate an immune response and also prolong period of protection (Glenn and Kenney, 2006; Nicolas and Guy, 2008). A study in pig indicated that the ID vaccinated pigs with 1/10 dose were protected against clinical FMD (Eble et al., 2009). To improves vaccine administration efficiency and to reduce the amount of vaccine per dose, the ID vaccination is a potential route for FMD vaccine spare in Thailand. However, immune response of FMD vaccination ID route has not been reported. Therefore, the study of proper dose of intradermal FMD vaccination produced by DLD is encouraging especially the study in a dairy cow.

An automatic needle-free injection device has been introduced to vaccinate cattle in 2000s. The needle-free injection is able to reduce labor for the animals restraint and reduce side effect of injection such as poor quality

of carcass on surrounding area of the injection site (Weese and Jack, 2008; Hickling et al., 2011). Moreover, the needle-free device can rapidly delivery vaccine compared with conventional method and reduce the risk of accidental injection in animal (Kale and Momin, 2014). However, there is a few studies in the application of the needle-free device in cattle. For example, Hollis et al. (2005) suggested that the vaccine of bovine respiratory disease vaccine can be administered to the animal via the needle-free device without any significant difference. The study on *Brucella abortus* RB51 vaccine in cattle (Pires et al., 2007) using needle-free device compared with needle syringe vaccination resulted in the similar enhanced immune response.

According to reviewed studies, the use of automatic needle-free vaccine via ID delivery system is promising as an alternative vaccine administration. This method have a potential to develop as a vaccine dosage reduction strategy and also stimulate the similar immune response comparing to conventional method with less restraint animal for vaccination. Therefore, the alternative route of FMD vaccination produced by DLD promises especially the study in a field environment.

The purpose of this study is to compare the immune response of FMD vaccine via intradermal injection, intradermal needle-free device, subcutaneous needle-free device and subcutaneous route (conventional method).

Objective of study

1. To study a proper dose of intradermal vaccination of FMD to stimulate an immune response in calves.

2. To evaluate the immune response of the different routes of administration; intradermal, needle-free intradermal and subcutaneous route of FMD vaccine produced by DLD in calves and heifers.

Hypothesis

Intradermal injection and needle-free intradermal injection can reduce the required dose of FMD vaccine, substitute the subcutaneous route (conventional method) and stimulate immune response equivalent to the subcutaneous route in dairy cattle.

Keywords: dairy cattle, foot and mouth disease, intradermal vaccination, needle-free device, subcutaneous vaccination

Advantages of study

- The application of an intradermal vaccination by an automatic needle-free device can reduce the amount of vaccine usage.
- This research could be used to demonstrates the effectiveness of the needle-free vaccination in which it is able to produce a similar immune response of FMD vaccine at a reduced dose administrated labor usage in restraining animals.

Chapter 2: Review literature

2.1 Foot-and-mouth disease

2.1.1 Overview

FMD is a viral disease caused by a single-stranded positive-sense RNA virus belonging to the genus *Aphthovirus* in family *Picornaviridae*. The diameter of FMDV particle is 25-30 nm with roughly spherical shape, which consists of the RNA genome with a protein shell or capsid surrounding (Mahy, 2004). The capsid contains 60 copies of capsomers. Each capsomer composes of four structural polypeptides, such as VP1, VP2, VP3, and VP4. The VP4 is entirely internal to the viral particle, while the VP1, VP2, and VP3 are on the surface and represent the antigenic properties of the virus. Genetic characterizations of FMDV strains are in the VP1 coding region with different nucleotide sequences in which are important for viral attachment and entry. Moreover, the immunity response is specific to a distinct serotype (Jamal and Belsham, 2013). FMDV exists as seven genetically distinct serotypes namely O, A, C, SAT1, SAT2, SAT3, and Asia1 (Domingo et al., 2002). Each serotype has a spectrum of variants with their own antigenic, biological and epidemiological characteristics (Domingo et al., 2002; Alexandersen et al., 2003; Jamal and Belsham, 2013).

The FMD is highly contagious transmissible disease, which causes high morbidity with low to moderate mortality. The disease affects cattle, buffalo, pig, sheep, goat, and seventy wildlife species, e.g. African buffaloes *(Syncerus caffer*). FMD has resulted in devastating livestock industries due to rapid spread and affect severe production losses. FMD has been considered as one of the most important diseases according to the international trade regulation.

The pathogenesis of FMD that reported by Rodriguez and Grubman (2009), FMDV replicates firstly at pharynx region after exposure via aerosol transmission. The virus invades the bloodstream within 24-48 hours. The clinical signs of FMD, in the adult animal, can be characterized by fever and presence of painful related to vesicles especially at the epithelia of the tongue, lips and mouth. The vesicle or blister like a lesion is also found on teats and between the hooves. In the adult animal, fatality is rare whereas the mortality in a young animal is often high due to myocarditis or starvation because of the lack of milk in infected dam. Due to the lesions appear in the mouth and feet of the infected animal in which the suffered animals refuse their food and water causing weakness and loss of productivity (Alexandersen et al., 2003). Most of the recovery affected animals become weakened and debilitated.

In general, FMD leads to the severe on animal productivity, constraints on international trade and rural poverty in developing country. There are considered great of economic losses at the farm and national level. There is a disruption of livestock production including reduced the milk production by 80% for chronic infection (Barasa et al., 2008). The disease causes the increased prices of livestock products and increased demand for livestock product importation. In order to control FMD at the farm or national level, losses of direct and indirect costs were budgeted such as the compensation to farmers, the labor cost of veterinarians, support personnel and general business disruption (Knight-Jones and Rushton, 2013).

Thailand is in an endemic area of FMD. The circulating virus serotypes consist of serotype O, A and Asia1 (Cleland et al., 1996). Economic losses including lacking the opportunities of livestock exportation, the cost of diagnosis, and farmers losses cash incomes. The losses cash incomes of farmers composed of the direct losses from low productivity, infertility,

mortality and the indirect losses from diagnostic test, prevention and control cost. The overall of economic losses resulted in increased the cost of production. Moreover, there were lost an opportunity of improvement of livestock industry and reduced food security resulted in the opportunity cost that is loss a huge number cost due to losses of the development of international trade.

2.1.2 FMD in Thailand

The FMD outbreak situation in Thailand was a limited number of reports. The FMD outbreaks in Thailand was investigated during 2005-2009 (Polratana, unpublished) and found that FMD outbreaks occurred 92, 45, 35, 52, and 50 times respectively in 2005 to 2009. Seekhaow and Intarapuk (2009) reported the prevalence of FMD infection by detection of non-structural protein for 14.49% and 3.67% in Kanchanaburi Animal Quarantine Station in 2006 and 2007 respectively. In 2011-2013, Inchaisri et al. (2015) reported that the FMD outbreak in Thailand have occurred with a higher frequency outbreaks in the same areas each year. For examples, the outbreaks in the western region were found a higher number of reports in Photaram district and Banpong district in Ratchaburi province, Thamaka district and Thamoung district in Kanchanaburi province, Kamphaengsang district in Nakornpathom province. In the northern region, the outbreaks occurred more frequency reports in Martha district in Lampoon province, Maewang district in Chiangmai province and Maelao district in Chiangrai province. In the central region and northeastern region occurred with a higher number of reports in Muakleg district in Saraburi province, Phatthananikom district in Lopburi province and Pakchong district in Nakorn Ratchasima province (Inchaisri et al., 2015). So, there were several outbreaks occur continually. These causes of an outbreak may associate with slow action to control an outbreak, type of animals in each area, high density of the animals in the area and the discipline of

protective immunity against FMDV (Parida, 2009). In order to reduce several outbreaks, the policy of disease controls needs to be intensively revised.

2.1.3 Control and prevention of FMD

It is crucial to prevent and control FMD for reducing the incidence of the outbreak. The elements of FMD control comprise immunity against FMDV from vaccination, quarantine or stamping out, animal movement restriction, biosecurity, and sanitation. In the outbreak situation, the epidemic area need to declare, restrict animal movement in the area, disinfectants were used to destroy the agent, emergency vaccination with high potency FMD vaccine were conducted in this area and ring vaccination was performed around the outbreak (Doel, 2003; Cai et al., 2014).

For the condition such as a high density of animal area and illegal movement, the vaccination must always combine with other control measures that limit the disease spread, for example, an intensive biosecurity, legal animal movement and use of disinfectant to reduce the agent into the farms. Spread between herds may not be controlled using only vaccination.

In the high-risk area of FMD outbreak in Thailand such as Saraburi, Nakorn Ratchasima province due to the high density of dairy cattle farm (Inchaisri et al., 2015), the measures to control the FMD included the use of FMD vaccine with regular immunization, reduced the agents with disinfectants and improved the biosecurity in the dairy cattle farm to reduce the incidence of FMD outbreak.

The control and prevention strategy in Thailand, DLD has established the policy. The policy was adopted with a developed national control plan along with neighboring countries through South-East Asia and China Foot and Mouth Disease campaign (SEACFMD). The SEACFMD roadmap is aiming to

eradicate FMD in 2020 (Sumption et al., 2012). DLD imposed policy by application of the Progressive Control Pathway (PCP). Thailand is in the risk-based area. In the risk-based FMD area, there needs to implement the vaccination campaign, active sero-surveillance and animal movement control (Jamal and Belsham, 2013). The vaccination campaign is recommended by DLD in which the farmer has been suggested to vaccinate their animals 2-3 times per year. The primary vaccination series of cattle including two primary doses at 4-6 months old.

2.1.4 FMD vaccine

Vaccination plays an important role to control the FMD. The predominant method of preventing FMD in endemic regions is mainly the regular vaccination with inactivated vaccine (Ana et al., 2004; Madhanmohan et al., 2010; Cai et al., 2014). The first FMD inactivated vaccine was developed from the virus collected from the epithelium and vesicular fluid of tongues of infected cattle and subsequently inactivated with formaldehyde (Waldmann et al., 1937). However, the disadvantage of inactivation by formaldehyde was incomplete viral inactivated (Barnett and Carabin, 2002). The binary ethyleneimine (BEI) was introduced in which used to solve this problem (Doel, 2003). BEI is the widest inactivation process, while the formaldehyde inactivation cannot reach the acceptable level of inactivation (Rodriguez and Grubman, 2009). Currently, FMD vaccine is an inactivated-whole virus preparation that is produced in cell culture and inactivated by BEI, then formulated with adjuvants (Cai et al., 2014). Adjuvants with inactivated FMDV is also important for vaccine potency. The antigen is usually mixed with aqueous adjuvant in ruminant formulation or oil adjuvant for pig formulation (Doel, 2003). Seed virus is used to infect a suspension cell culture, inactivated with BEI and concentrated. In term of the selection of vaccine strains, the producers have to monitor continuously the current situation and testing the

appropriate of strain of virus for vaccine production (Doel, 2003; Keeling et al., 2003; Kitching et al., 2008).

The vital role of FMD vaccine is serotype specific and no cross-protection between strains (Pacheco et al., 2010), therefore the selection of virus strains are essential to the epidemiological study in the area (Doel, 2003). In term of vaccine quality assurance including the correct choosing the virus strain for FMD vaccine production and the standard procedure should be followed in the Terrestrial Manual by OIE (Kitching et al., 2008). Vaccine delivery, packaging, cold chain, and logistics management affect quality of FMD vaccine. The vaccine delivery should be correctly stored at temperature 2-8 °C. However, there are several factors of cold chain and logistics management have to control such as equipment, procedures, labors, and vehicles for preventing vaccine damage (Ferrari et al., 2016). The inappropriate temperature destroys the stability of structural proteins FMDV including the integrity of 146S particle (Doel, 1996).

In Thailand, DLD reported that the predominant strain of FMDV was serotype O followed by serotype A and Asia 1 (Gleeson et al., 1993; Knowles et al., 2012). In the present, the FMD vaccine in Thailand is the trivalent (FMDV O, A and Asia1 serotype) manufactured by DLD. A Regional Reference Laboratory in Thailand which recognized and collaborating with OIE is routinely checking a matching of vaccine with virus field strain.

2.1.5 Vaccine application

The target for vaccination is the susceptible animal such as animal that lose any maternally derived antibodies. The duration of protective immunity has been involved by vaccine potency, vaccine matching and prior immunity from vaccination (Ferrari et al., 2016). The vaccination schedule should be provided by the vaccine manufacturer. FMD vaccines provide

relatively short-lived protection (Rodriguez and Grubman, 2009). Inactivated FMD vaccine is providing an immunity for three months (Barteling and Vreeswijk, 1991). However, the high potency vaccine can provide an immunity lasts for 6 months (Cox et al., 2003). Due to the type of vaccine and the possible factors such as the single vaccination gives the short-lived protective levels of the antibody, the best primary courses of vaccination are required two doses of vaccine at least one month apart (Pay, 1985). The FMD vaccination regularly requires repeated vaccination in order to maintain protective immunity levels.

The vaccine administration should be properly performed according to the manufacturer. The conventional route of administration recommend by Brückner and Saraiva-Vieira (2010) is a subcutaneous route (SC) on the prescapular area in ruminants. The animal immune response is depended on site of antigen deposit, the time since the previous vaccination, and the number of vaccine doses in the lifetime. The length of immunization, duration of exposure and the challenge methods are critical for animal clinical protection (Parida, 2009). Therefore, the method of vaccinating should be administrated carefully with a standard operating procedure.

Thailand is an endemic area of FMD. The vaccination program should be set to reduce the clinical outbreaks of FMD. For FMD prevention strategy, the DLD has recommended that cattle must be initially vaccinated at age of 4-6 months, and animals should receive a second booster vaccination after a month later. The national annual vaccination program is every 4-6 month. FMD vaccination procedure is advised to inject vaccine via SC with 2 mL per dose in dairy cattle.

2.2 Implications of vaccination

Even though vaccination is conducted routinely in Thailand, the outbreak has often reported. The study showed the low herd immunity in beef cattle (50.24% for serotype A, 51.81% for serotype O and 51.05% for serotype Asia1) (Jithlang and Sirimongkorat, 2008) . However, low immunity in protection may be caused either by the failure to vaccinate or by the actual vaccine failure. The vaccination failures may be due to vaccine failure including vaccine strain unmatching with viral field strain, poor quality batch from vaccine factory and cold chain management. And due to failure to vaccine. FMD vaccine probably administrated with the wrong procedure, denying of farmer to vaccinate their animal due to the difficulty animal restraint and insufficiency of vaccine quantity (Pay, 1985; Doel, 1996; Keeling et al., 2003; Elnekave et al., 2016b).

There was a problem of the number of doses of vaccine distribution to the animals in Thailand. Data from DLD showed that DLD provided for free of charge annually approximately 11 million doses of FMD vaccine for ruminants while DLD produces and sales about 35 million doses of FMD vaccine for pig populations. In 2015, the ruminant population was reported about 5 million for beef cattle, 600,000 for dairy cattle, 1 million for buffaloes, and 460,000 for goats (Office of Agricultural Economics, 2016). According to DLD recommendation, ruminants with the regular vaccination 2-3 times a year, a number of vaccination doses may not be sufficient for susceptible population. The vaccine coverage can be used to an indicator of the performance of the distribution (Ferrari et al., 2016).

2.2.1 Vaccine coverage

The proportion of qualified cattle that are actually vaccinated is termed vaccine coverage (Ferrari et al., 2016). In general, at least 85% of the

susceptible population should be vaccinated to achieve a herd effect of protective immunity for prevention and control FMD (Doel, 1999). The vaccine coverage is essential to control FMD due to the rate of the viral spread (Ferrari et al., 2016). The lack of the vaccine coverage due to only a few animals being vaccinated results in the poor population immunity.

2.3 Route of administration

The route of administration is a critical factor for a success of immunization. The most common route of FMD vaccine administration is the subcutaneous (SC) or intramuscular (IM) injection (Pay, 1985; Doel, 1999). The route of vaccine administration is designed to improve the protective immune response and to reduce side effects of vaccine (Ada, 1990). The lipophilicity is the main influenced factor of chemical distribution (Jerzsele, 2012) in order to stimulate immune response in which SC has excess lipid accumulation.

Currently, dermis and epidermis become alternative sites for prophylactic vaccination because the areas are rich in efficient antigen-presenting cells, that are able to induce protective immunity (Nicolas and Guy, 2008). The numerous immune cell is dendritic cells that antigen present to initiation of adaptive immune response (Van Drunen Littel-van den Hurk, 2006; Pandya et al., 2012). The benefits of ID vaccination are to reduced dose and increase immune enhancement (Glenn and Kenney, 2006). Due to an ID delivery of vaccine antigen rather than SC or IM, it can stimulate equivalent to or superior the protective immune response with a smaller quantity of vaccine antigen (Hunsaker and Perino, 2001).

Recently, several studies have investigated FMD vaccination by using ID route. Eble et al. (2009) and Pandya et al. (2012) suggested that ID vaccination against FMD is suitable as an alternative route. Eble et al. (2009) indicated that ID route is a good alternative, as ID application induce a very

efficient immunological response against FMD. This study found that ID vaccinated pigs with 1/10 dose were equally protected against clinical disease and subclinical virus shedding as IM vaccinated pigs with a full dose (Eble et al., 2009). Furthermore, the dose required by the ID route is even lower than SC route.

In order to increase the quantity of available vaccine for the ruminant at the current DLD vaccine production. we proposed that ID vaccinated dairy cattle can be the alternative potentially vaccine delivery. However, there is lack of information on the application of FMD vaccine produced by DLD via ID route to the immune response against FMD.

2.4 Automatic needle-free device

Currently, the automatic needle-free device is using widely in the human and is introducing in the animals. Comparison with the conventional method, the standard method of vaccine antigen injection with needle-based is an invasive method of administration (Weese and Jack, 2008). The needle-based injection is usually through IM and SC which might damage surrounding tissue, for example, IM injection showed 1.3% of carcass visible injection site including lesions, scar and reduce tenderness (Van Drunen Littel-van den Hurk, 2006) and also resulted in involuntary losses by meat trimming. Moreover, injection by reused needle or incorrectly used needle can transfer the disease especially blood-borne infectious disease, such as bovine leukosis and anaplasmosis (Hollis et al., 2005; Kale and Momin, 2014). The accidental injury of individual handling or broken needle fragment in the animals might occur during administration by needle-based vaccinating (Weese and Jack, 2008). Automatic needle-free device deals significant advantage compared to conventional needle-syringe methods, the device was introduced and widely used in human over 50 years (Daniels and Headquarters, 2014).

The advantages of automatic needle-free injection include the reduction of utilizing the needle, time and labor. Blood-borne disease can be prevented by using the automatic needle-free device (Rao et al., 2006; Weese and Jack, 2008), for example, blood-borne infection as *Anaplasma marginale* (Reinbold et al., 2010). The needle-free injection was first described by M. (1936), who used in his patient by jet injection. The principal of jet injector or needle-free injection is using a high-velocity liquid flow to deliver vaccine through tissue (Kale and Momin, 2014; Chen et al., 2017). It is powered by compressed carbon dioxide (CO_2) as the power source for the needle-free device, using a shin-tenting technique (Hollis et al., 2005; Harris et al., 2006). The automatic needle-free vaccinating techniques delivers vaccines either SC or IM by adjusting pressure, for example in the Pulse 250 system, according to the manual, for injection in the intradermal route is used 40-50 PSI (pound per square inch), 45-55 PSI for SC and 60-65 PSI for IM.

The use of automatic needle-free injection in pig indicated that this method can reduce the transmission of porcine reproductive and respiratory syndrome virus due to needle-syringe procedure (Otake et al., 2002). Thus, the needle-free technology can reduce lateral transmission of disease. For an apply automatic needle-free device via ID resulted in improved immune response with minimal antigen doses and the total number of animals that could be vaccinated from one batch can be increased (Pandya et al., 2012). The immune response could be improved with a higher dispersion rate to the skin from the device (Chen et al., 2017). The weakness of this method is not applicable for the intravenous route (Daniels and Headquarters, 2014; Kale and Momin, 2014).

This method has been used successfully to vaccinate cattle. There are several studies in which needle-free vaccination has been applied. For example, Rey et al. (2013) showed that both needle-free and needle-syringe

revealed a significant antibody response, in which needle-free resulted in a lesser of skin reaction and adverse carcass quality effect. Chen et al. (2017) suggested that needle-free injection system resulted in the lower rate of adverse animal reaction and high injection speed resulted in enhance of vaccination efficiency. In addition, Van Drunen Littel-van den Hurk (2006) found that needle-free delivery system through intradermal can be effective for vaccination with a *Bovine Herpes virus type 1* DNA vaccine. Hollis et al. (2005) also performed the needle-free in calves with *Infectious Bovine Rhinotracheitis Virus* Vaccine (IBR), *Mannheimia haemolytica* Bacterin-Toxoid and *Leptospira pomona* Bacterin and Pires et al. (2007) evaluated the needle-free vaccinating with *Brucella abortus* RB 51 vaccine.

Furthermore, in term of intradermal with the needle-free system, Pandya et al. (2012) stated that the needle-free device can be substituted to conventional method. The study suggested that the effective protection against FMD can be achieved with 1/16 of the recommended vaccine dose when delivered using the needle-free (Pandya et al., 2012). The results showed that cattle vaccinated with 1/16 and 1/4 dose using the needle-free device were protected when challenged at 7 and 28 days after the vaccination (Pandya et al., 2012). We suggest that the immune response of the automatic needle-free device via ID attribute to practical and potentially effective of vaccinating technique in dairy cattle. The device provides at least an equivalent tool improving immune response compared with needle-syringe injection. However, there is no information on the study of this device in dairy cattle comparable to conventional method in Thailand. Therefore, the automatic needle-free vaccination of FMD vaccine produced by DLD via ID in this study is especially encouraging the study in a dairy cow.

2.5 Immune response to FMD vaccination

Types of immunity compose of innate immunity and adaptive immunity. The innate immunity is presented from birth which is non-specific immune responses, whereas the adaptive immunity is antigen-specific and provides greater efficiency to the exposure. The innate immunity plays important role in the initiation and direction of an adaptive immune response (Janeway et al., 2001). However, the vaccination is active immunization resulted in the adaptive immunity (Doel, 1999) The adaptive immune products are effective against the specific challenge (Iwasaki and Medzhitov, 2010). The two types of adaptive or specific immunity are humoral immunity and cell-mediated immunity.

The development of systemic antibody response after vaccination in which assessed as for the immunoglobulin isotype composition. In the study of Capozzo et al. (1997) stated that the immunoglobulin G1 (IgG_1) response predominates over IgG_2. After the vaccination, IgM was firstly in peripheral blood for two to four days (Elzein and Crowther, 1981) and reaching a peak between five to ten days (Cowan, 1973) and followed by IgG_1 which was associated with neutralization. IgG presents from four days and peaks between fifteen and twenty days of vaccination (Cowan, 1973; Elzein and Crowther, 1981). There was a study about the neutralization of virus, and the neutralization was associated with IgA, IgG in the steer after the third dose of aluminum hydroxide/saponin regular FMD vaccine (Garland, 1974).

The vaccinated animals develop immune responses called antibody, which is produced by B cells in the part of humoral immune responses (Janeway et al., 2001). Antibodies are stimulated by T helper cells in which working for neutralization. Antibodies can be called as Immunoglobulin (Ig) which are glycoprotein molecules produced by plasma cell forming the B

cell. The various antibodies are classified with isotype, for example, IgA, IgD, IgE, IgG, and IgM. However, the specific immune system responses directly attribute to the cytotoxic T-cells and T helper cells which are cell-mediated immunity. Childerstone et al. (1999) showed that the cell-mediated immunity can clear the virus. Vaccinated animals provide the humoral B cell responses and cell-mediated T cell responses. In the initial vaccinations, antibodies produce from differentiating B cell which mostly is IgM. Nonetheless, IgM has a short-lived immune (Janeway et al., 2001). After the second vaccination, the enormous populations of memory cells are activated, which T cells and B cells give a high yield of memory cells producing the IgG (Ada, 1990)

ID vaccinated animals induce the immune response from dendritic cell (Hunsaker and Perino, 2001). Dendritic cell acts as processing antigens and present to T-cells (Liard et al., 2012). Therefore, an enormous of dendritic cell can induces a high level of immune response resulted in high antibody.

2.6 Measuring antibody response to FMDV

Evaluation of the immune response to vaccination is an important method for determination of vaccine effectiveness. Vaccines composed of purified structural proteins of FMDV (Doel, 2003). The vaccinated animals only elicit antibodies against the structural protein (SP) (Pega et al., 2015). Whereas, the infected FMDV animals produce antibodies against the SP and non-structural protein (NSP) of the virus (Doel, 2005). According to the manual of diagnostic tests and vaccines for terrestrial animals (Kitching et al., 2008; OIE, 2017), the serological test suggested to detect antibodies resulting from vaccination using SP tests is to be used monitoring population immunity (OIE, 2017), while NSP test is used to differentiate between infected and vaccinated animals (OIE, 2017).

Serological tests for FMD comprise two types; SP and a NSP test. Due to the scenarios of antibody response, animals in which infected is elicited antibody to SP and NSP on 3-5 days after infected, reached the high level on 14 days after infected (Alexandersen et al., 2003). Moreover, antibody of both can be detected on 665 days after infected (Moonen et al., 2004). NSP antibodies are developed only in infected animals with all serotype of FMDV. For the endemic regions of FMD, the NSP test is provided to identify the previous or present viral replication in the animal and to confirm suspected cases to virus circulation (Cai et al., 2014), but the NSP test is not serotype specific. For substantiating freedom from infection, the NSP test can be confirmed (OIE, 2017).

The SP tests are serotype-specific and detect antibodies elicited by vaccination and infection, so these tests can be used to demonstrate the efficacy of vaccination (De Jong and Bouma, 2001). To determine the level of antibody response to vaccination must be demonstrated the immune response against homologous virus antigen of FMD vaccine (Doel, 2003). The measurement of immune response to FMD vaccination combined with an appropriate of vaccination schedule can be reduced the outbreak of FMD.

The SP tests are the virus neutralization test (VNT), solid-phase competition ELISA and liquid phase blocking ELISA (LP ELISA). There are highly sensitive resulted from the virus or antigen used in the test that is closely matched to the strain circulating in the field. The advantages of ELISA are using serotype-specific polyclonal antibodies, so they are not dependent on tissue culture system. The diagnostic time is quicker than VNT (Alexandersen et al., 2003). However, the false positive reactions can be expected in a small proportion of the serum (OIE, 2017). The recommendation is used the ELISA to screening. The confirmative test of the positive sample using VNT can be minimizing the false positive results (Ma et al., 2011). The VNT relies on tissue

culture and is more prone to variability than ELISAs. The study found that VNT is more accruable than SP-ELISA because of neutralizing method for the detection of antibodies against FMDV structural proteins (Manzoor et al., 2015). VNT demonstrates on the inhibition of virus infectivity in cell culture of neutralizing antibodies in serum (OIE, 2017). Currently, OIE considered VNT as the gold standard for detection of antibodies to SP antibodies (OIE, 2017).

Interpretation of VNTs can vary among laboratories in which depend on the negative/positive cut-off threshold. In Thailand, according to Quality Control of Vaccine Production unit, Bureau of Veterinary Biological. DLD, Pakchong, Nakorn Ratchasima province, there established their criteria followed by OIE (2017). Titers are determined by the presence or absence of cytopathic effect (CPE) with a direct microscopic examination of the plate test wells for the presence of the viral antigen in the cell monolayer (Maradei et al., 2008). According to OIE (2017), a titer of 1/45 or more of the final serum dilution in the serum/virus mixture is considered as positive. A titer of less than 1/16 is regarded to be negative. For certification of individual animals for the purposes of international trade, titers of 1/16 to 1/32 are regarded to be doubtful, and further serum samples may be requested for testing; results are regarded to be positive if the second sample has a titer of 1/16 or greater.

Chapter 3: Materials and Methods

3.1 Animals

This study was performed in a large farm with 1,200 Holstein Friesian cows located in the Saraburi province, Thailand. FMD vaccination program in this farm is routinely administered three times per cow per year. Whole herd vaccination has been executed at the same week. First-time vaccination for calf is given when calf is older than 4 months.

The experiments were conducted in 40 male calves (experiment 1) and 40 heifers (experiment 2.) The male calf with age range more than 4 months with no prior FMD vaccination were random selected in one batch for our study. In heifer with random selection in one batch, the age was range 13-15 months old with receiving at least two vaccinations before beginning the experiment. Animals were identified by unique ear-tag numbers on left ears at the beginning of experiments. Before beginning of experiments, the blood sample of all experimental animals were collected to determine the level of antibody to set as the base line before vaccination. In addition, to monitor infection status in this farm, blood samples of calves were selected for 98 samples of 25 calves to measure the level of antibody against nonstructural protein of FMD virus.

The FMD clinical signed was continuously observed during study periods. Both of experimental calves and heifers were examined to detect FMD clinical signs and adverse effect after vaccination by a veterinarian of this farm.

3.2 Needle-free device

The Pulse 250 Needle Free Systems model (Pulse®, USA) was used in this study. The device was calibrated as a routine maintenance procedure by agency company before beginning the experiment. The device can delivery vaccine for 3 levels of 1, 2 and 5 mL with powered by compressed carbon dioxide (CO_2). The

vaccine is administered through a high-pressure hose towards a handpiece with 0.35 mm diameter. The pressure was adjusted to 45 PSI for the intradermal route in the calves, while the heifer was used 50 PSI for the intradermal route and 60 PSI for the subcutaneous route.

3.3 Vaccine and administration technique

The experimental animals were vaccinated with killed trivalent vaccine (O, A and Asia-1 serotype, DLD) containing with at least three times of dose that protects 50% of the animals challenged (PD_{50}), with alum gel as adjuvant. The same vaccine batch was used for both experimented in calves and heifers (T60D), which contained FMD virus strain of O189, A sakolnakorn and Asia-1.

The experimental animal was restrained with a squeeze chute in the gate and tagged ID using ear tag plastic at left ear between tip and base avoiding cartilage and blood vessels. To avoid any kind of contamination, a vaccinator put on medical gloves before vaccination. The injection site was disinfected by 70% alcohol before vaccination. The conventional SC injection was conducted into the loose area above right scapular area. The SC injection was performed in the center of right triangle, using a non-dominant hand to pinch skin, after that angle the needle at 30 to 45 degrees from the surface of skin before injection into skin fold. Manual ID was performed at the right neck of animals with a needle inoculation into the intradermal layer, with needle size 21-gauge 1 inch long. The manual ID administering was performed firstly by using the non-dominant hand to pull the skin taut to ensure easy penetration of needle. Secondly, a vaccinator held the needle at a 5 to 15 degree angle parallel to the skin before injection and releasing vaccine. The automatic ID was performed with a needle-free injection device (Pulse® 250 Needle Free Systems, USA) on the right side of the neck using nozzle face to drive vaccine into the animal.

3.4 Ethical approval

This study was approved by Chulalongkorn University Animal Care and Use Committee in accordance with university regulations and policies governing the care and use of laboratory animals. The animal use protocol number was 1731078.

3.5 Study design of experiment 1 in calf

Male healthy calves were selected randomly from calf population (Table 1.). Forty male calves were randomly allocated into seven groups of five calves each (except for 10 calves in group II) and each group was administered trivalent killed FMD vaccine at different doses and administration routes (Table 1).

The animal sample size was assigned following the foot and mouth disease vaccination and post-vaccination monitoring guidelines, published by The Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) that recommend the sample size of clinical trials to evaluate the vaccine quality in vaccinated cattle is five calves per group (Ferrari et al., 2016).

Table 1 The experimental designs in calf with different doses and routes of trivalent killed FMD vaccine (O, A, Asia-1) administration allocated into seven groups.

Group	Numbers of calf	Dose volume (mL)	Type of solution	Route of administration
I	5	1	Normal saline	ID
II	10	2	Vaccine	SC
III	5	1	Vaccine	ID with needle-free device
IV	5	0.25	Vaccine	ID
V	5	0.5	Vaccine	ID
VI	5	1	Vaccine	ID
VII	5	2	Vaccine	ID

Saline = normal saline solution as a non-immunized control. Vaccine = Trivalent killed FMD vaccine

3.6 Study design of experiment 2 in heifer

The forty Holstein Friesian healthy heifers receiving at least 2 FMD vaccinations before experiment were randomly allocated into four groups equally. The first group was vaccinated by the conventional method with 2 mL via manual SC route. The second group was vaccinated 2 mL with the automatic needle-free device via SC route. The third group was vaccinated 1 mL via manual ID route with the needle and the last group was vaccinated 1 mL with the automatic needle-free device via ID route. (Table 2.).

The sample size was designed by the recommendation for the clinical trials to evaluate the immunity in a heifer or cow for the foot and mouth disease vaccination and post vaccination monitoring guidelines (Ferrari et al., 2016).

Table 2 The experimental design in heifer with different routes and doses of trivalent killed FMD vaccine (O, A, Asia-1) administration allocated into four groups.

Group	Numbers of heifers	Dose volume (mL)	Route of administration
I (control)	10	2	SC
II	10	2	SC with needle-free device
III	10	1	ID
IV	10	1	ID with needle-free device

3.7 Serum sampling

Blood samples of all experimental animals were collected from the jugular vein on 0 and at 7, 14, 21, 60, 86 and 120 days post vaccination (dpv). Serum samples were identified and stored at -80 ˚C until subsequent analysis.

3.8 Serological test

3.8.1 Non-structural protein test

The samples were collected from 25 calves which were selected five calves from one control group (group II) and five per group from four treatment groups (group IV, V, VI and VII) on day 0, 7, 14 and 21 dpv to determine the natural infection of FMDV.

The serum was tested for the presence of antibody that was specific to FMD NSP using FMDV PrioCHECK® NS blocking ELISA (Thermo Fisher Scientific Inc, USA) in order to monitor the natural infection from FMDV. The procedure was performed following the manual of ELISA for antibody detection against non-structural protein of FMDV in serum of cattle, sheep, goats and pig of PrioCHECK® NS blocking ELISA (OIE, 2017). For the interpretation of the percent inhibition, the value greater than or equal to 50% was considered as positive result for the presence of antibodies against the NSP of FMDV in the sample.

3.8.2 Virus neutralization tests (VNTs)

The neutralizing antibody titer (NAT) in serum was measured by a virus neutralization test (VNTs) at Quality Control of Vaccine Production unit, Bureau of Veterinary biological. Department of development livestock, Pak-Chong, Nakorn-Ratchasima province, Thailand. The procedure was performed following the OIE procedure standard of the VNT for FMD (OIE, 2017). The homologous vaccine strains were used for VNTs. A serum sample was inactivated at 56 °C for 30 minutes in a water bath before VNT tested. The VNT was performed with lamb kidney cells in a flat-bottomed tissue-culture grade microtiter plate. Stock virus was grown in cell culture monolayers and stored at –20°C after the addition of 50% glycerol (virus had been found to be stable under these conditions for at least 1 year). The control standard serum was post-vaccination serum including positive and negative control. A suitable used medium was Eagle"s complete medium/LYH with HEPES buffer and antibiotics.

In brief of VNT procedure, sera were diluted in two-fold dilution series across the plate using two rows of wells with volume 50 µl. The dilution was started from a 1/4 dilution, sera were diluted in a twofold with medium, dilution series across the plate, using two rows of wells per serum. The previously titrated virus was added; each 50 µl unit volume of virus suspension should contain about 100 TCID50 (50% tissue culture infective dose) and incubate at 37℃ for 1 hour with the plates covered. A volume of 50 µl of cell suspension was added to each well with cell suspension at 10^6 cells/mL. Plates were sealed with pressure-sensitive tape and incubated at 37℃ for 2 days and then readings the plates. Neutralizing antibody titers were expressed as the log 10 serum dilution neutralizing 50% of the virus inoculums (100 $TCID_{50}$).

Positive wells (where the virus had been neutralized and the cells remained intact) were seen to contain blue-stained cells sheets (no cytopathic effect (CPE)); the negative wells (where virus had not been neutralized) were empty (CPE). Titers were expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells were protected (Kärber, 1931). The test was considered to be valid when the amount of virus used per well is in the range log10 1.5– 2.5 TCID50, and the positive standard serum was within twofold of its expected titer.

To determine the protective level of the antibody, the neutralizing antibody titers were expressed as the log 10 serum dilution. The criteria that a titer of 1.65 or more than of the final serum dilution was considered as positive, while a titer of less than 1.20 was regarded as negative. On the other hand, titers between 1.20 to 1.50 were regarded that doubtful. The further testing is required, if the second-round testing had a titer of 1.20 or greater result is considered as positive (OIE, 2017). However, in this study because of the procedure of the laboratory, the result below 1.65 were considered as negative of protective level.

3.9 Data analysis

Data on the experiment were accessed and summarized using SPSS version 22 (IBM, New York). The neutralized antibody titer was presented in log 10. After analysis, the NAT was estimated by a generalized linear mixed model (GLMM) and presented as average NAT with standard error (SE). Bonferroni pairwise comparison was determined to compare the estimated NAT among groups and compare among sampling time within group.

The different average of NAT was analyzed using linear regression in GLMM. The NAT were set as the response variable, the animal as random variables and sampling time and group as explanatory variable. To compare within experimental group, the estimated NAT after vaccination was compared among sampling time points. In order to compare among experimental groups, the estimated NAT in each sampling time was compared among groups.

When the neutralizing antibody was equal to or above 1.65, the sera were considered as protective immunity (OIE, 2017). The proportion above the protection threshold for VNT results were compared by a binary logistic regression with GLMM, calculated as percentage with SE. The data were converted to 1 as protective immunity and 0 as unprotective immunity and setting as the response variable. The animal was set as random variables whereas the sampling times and the groups were set as explanatory variable. To compare within experimental group, the estimated proportion of protective immunity after vaccination was compared among sampling time points. To compare among experimental groups, the estimated proportion of protective immunity each sampling time was compared among groups.

As for all analyses, a p-value <0.05 was considered as indicating of statistical significance. The error variance of all model was checked by plotting residual against predicted value of final model.

Chapter 4: Results

4.1. Clinical and laboratory assessment

In the experiment, all animals were in the same house and monitored the adverse effect of vaccination. All experimental animals have been examined the clinical signs of FMD during our study period. All animals did not show any adverse effect and clinical signs of FMD. All tested serum of selective calves for FMD NSP antibodies are negative. Therefore, there are no evidences of natural FMD infection in our experiment animals. For all regression models, the variance of the residual error was homoscedasticity constantly for all values of the explanatory variables.

4.2 Neutralizing antibody response in experiment 1 (calf)

The neutralizing antibody response of FMD vaccination in calves showed that they had the immune response after the primary and booster dose of trivalent FMD vaccine (O, A and Asia1).

4.2.1 NAT for serotype A

FMD vaccinated calves in all groups did not show the immune response to FMDV serotype A (table 3).

4.2.2 NAT for FMDV serotype O and Asia-1 in ID groups

There was the immune response in all vaccinated calves. The NAT in all ID groups did not show significantly difference among groups on 7 dpv. However, one week after the booster (day 14), the NAT of IV and VI groups increased significantly. The NAT against FMDV serotype O reached the highest level on 21 dpv in group IV (manual 0.25 mL) (table 4).

For serotype Asia-1, we found that group IV had an average NAT significantly reached the highest level on 21 dpv (table 5). This group had the significant difference in NAT from other groups, particularly in 21, 60 and 86 dpv (table 5, figure 4, 5, 6 and 7).

4.2.3 NAT for FMDV serotype O and Asia-1 using automatic needle-free device via ID

There was no significantly different of NAT against FMDV serotype O and Asia-1 between group II and group III ($p>0.05$) as shown in figure 2 and 3. The NAT levels reached the highest value in both serotypes on 21 dpv.

4.2.4 The proportion of protective level for serotype O and Asia-1 in calves

NAT equal to or above the 1.65 is considered as the protective level. The proportion of vaccinated animal that had reached the protective level was shown in table 6, 7 and figure 1, 2, 3, 4, 5, 6 and 7.

We found low proportion of protective immunity against serotype O in vaccinated animal except group IV on 21 dpv. However, there was no significantly difference among groups (table 6).

All groups had significantly increased on the proportion of vaccinated animals that reached the protective level against serotype Asia-1 on 21 dpv except the group I (table 7). The proportion of vaccinated animals in group II declined over 21, 60, and 86 dpv. However, in this group the significant difference was found on 120 dpv (figure 2). Group III, IV, V, VI and VII did not showed significantly difference over 21, 60, and 86 dpv (figure 3, 4, 5, 6 and 7).

4.3 Neutralizing antibody response in experiment 2 (heifer)

All groups of heifers, the NAT against serotype O, A and Asia-1 increased significantly on 7 dpv (table 8, 9 and 10).

4.3.1 NAT for serotype O, A and Asia-1

All heifer groups had NAT against serotype O significantly increased on 7 dpv (table 8), in which group III was significantly difference from the others. The NAT against FMDV serotype O, group I, III, IV did not show significantly difference on 7 and 14 dpv. The NAT against FMDV serotype O of group III was significantly on 120 dpv (figure 10).

An average NAT against FMDV serotype A was significantly difference on 7 dpv in all heifer groups. In addition, especially in group III and IV were significantly difference from group I and II on 7 and 14 dpv (table 9). On 7 dpv, only group III showed that an average NAT had reached the protective level (figure 9).

In all heifer groups on 7 dpv, the average NAT against serotype Asia-1 was significantly difference comparing with the day 0 (table 10). An average NAT against serotype Asia-1 reached the protective level on 7 dpv in group I, III and IV without significantly difference among groups (figure 8, 10 and 11). On 14 dpv, group III and IV showed that an average NAT reached the protective level in which were significantly difference from group I and II. There was no significantly difference on the NAT against serotype Asia-1 in group I, III and IV on 14 and 21 dpv.

4.3.2 The proportion of protective level for serotype O, A and Asia-1

The proportions of protective level of a heifer groups were significantly highest on 7 dpv for all serotype. There was no significantly difference among groups on every sampling time (figure 8, 9, 10 and 11).

In all heifer group, the proportion of protective level against serotype O was significantly difference on 7 dpv compared with day 0 (table 11). There were slightly decreased proportion of protection in all groups during the observation after 7 dpv.

On the serotype A, there was low proportion of protection on 7 dpv of all heifer groups. There was significantly difference on 7 dpv compared with day 0 (table 12).

The proportions of protection against serotype Asia-1 in all heifer groups were significantly difference on 7 dpv compared with day 0. After vaccination, the proportions of protection against serotype Asia-1 of group I, III and IV were slightly decreased after 14 dpv (table 13).

The ID groups had a higher proportion of protective immunity than SC groups with no significantly difference (table 11, 12 and 13). The proportion of protective immunity of needle-free device group did not differ from other groups (figure 8, 9, 10 and 11).

Table 3 The neutralizing antibody titers (log 10) against FMDV serotype A in calves (experiment 1).

Group	Neutralizing antibody titer against serotype A expressed as log10 (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
II	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
III	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
IV	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
V	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
VI	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
VII	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
Positive serum	2.78	2.78	2.63	2.63	2.63	2.78	2.78
(Accepted range)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)
Negative serum	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
(Accepted range)	(≤0.75)	(≤0.75)	(≤0.75)	(≤0.75)	(≤0.75)	(≤0.75)	(≤0.75)

Note; Group I; injected with NSS 1 mL via ID Group II; conventional vaccinated FMD vaccine 2 mL via SC, Group III; automatic needle-free vaccinated FMD vaccine 1 mL via ID, Group IV; vaccinated FMD vaccine 0.25 mL via manual ID, Group V; vaccinated FMD vaccine 0.5 mL via manual ID, Group VI; vaccinated FMD vaccine 1 mL via manual ID, and Group VII; vaccinated FMD vaccine 2 mL via manual ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling times ($p<0.05$).

Table 4 The neutralizing antibody titers (log 10) against FMDV serotype O in calves (experiment 1).

Group	Neutralizing antibody titer against serotype O expressed as log10 (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	0.93±0.09^{ABCD}	0.90±0.09	0.78±0.09^{A}	0.81±0.09^{A}	0.75±0.09	0.84±0.09^{A}	0.75±0.09
II	0.83±0.06^{ab,A}	0.78±0.06^{a}	0.81±0.06^{a,AE}	0.99±0.07^{b,A}	0.78±0.06^{a}	0.90±0.06^{a,A}	0.78±0.06^{ab}
III	0.78±0.09^{a,ABCD}	0.87±0.09^{a}	0.98±0.09^{a,AB}	0.78±0.09^{a,A}	0.75±0.09^{a}	0.75±0.10^{a,A}	0.75±0.10^{a}
IV	0.81±0.09^{a,A}	0.90±0.09^{a}	1.10±0.09^{ad,B}	1.47±0.09^{c,B}	0.81±0.09^{b}	1.11±0.09^{d,B}	0.87±0.09^{abd}
V	0.75±0.09^{B}	0.84±0.09	0.75±0.09^{A}	0.75±0.10^{A}	0.75±0.09	0.78±0.09^{A}	0.79±0.10
VI	1.10±0.10^{ab,C}	0.99±0.10^{a}	1.25±0.09^{b,D}	1.20±0.09^{c,C}	0.87±0.10^{a}	0.86±0.10^{a,A}	0.75±0.10^{a}
VII	0.78±0.09^{AD}	0.78±0.09	0.75±0.09^{E}	0.96±0.09^{A}	0.75±0.09	0.82±0.10^{A}	0.78±0.09
Positive serum (Accepted range)	2.10 (1.95±0.23)	2.10 (1.95±0.23)	1.725 (1.95±0.23)	1.725 (1.95±0.23)	1.725 (1.95±0.23)	1.80 (1.95±0.23)	1.80 (1.95±0.23)
Negative serum (Accepted range)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)

Note; Group I; injected with NSS 1 mL via ID Group II; conventional vaccinated FMD vaccine 2 mL via SC, Group III; automatic needle-free vaccinated FMD vaccine 1 mL via ID, Group IV; vaccinated FMD vaccine 0.25 mL via manual ID, Group V; vaccinated FMD vaccine 0.5 mL via manual ID, Group VI; vaccinated FMD vaccine 1 mL via manual ID, and Group VII; vaccinated FMD vaccine 2 mL via manual ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling times (p<0.05).

Table 5 The neutralizing antibody titers (log 10) against FMDV serotype Asia-1 in calves (experiment 1).

Group	Neutralizing antibody titer against serotype Asia-1 expressed as log10 (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	0.75±0.15	0.75±0.15	0.75±0.15	0.75 ± 0.15^{ABCDE}	0.75 ± 0.15^{A}	0.75 ± 0.15^{A}	0.75 ± 0.15^{C}
II	0.75 ± 0.10^{a}	0.92 ± 0.10^{b}	0.87 ± 0.10^{b}	$2.07\pm0.11^{c,BC}$	$1.77\pm0.11^{d,BC}$	$1.65\pm0.11^{e,C}$	$1.17\pm0.10^{b,A}$
III	0.75 ± 0.15^{a}	0.93 ± 0.15^{a}	0.75 ± 0.15^{a}	$1.77\pm0.15^{b,CDE}$	$1.20\pm0.15^{c,CD}$	$1.12\pm0.16^{a,A}$	$1.07\pm0.17^{a,C}$
IV	0.75 ± 0.15^{a}	$1.23\pm0.15^{b,B}$	1.05 ± 0.15^{b}	$2.10\pm0.15^{c,A}$	$1.41\pm0.15^{b,B}$	$1.83\pm0.15^{c,D}$	$1.26\pm0.15^{b,B}$
V	0.75 ± 0.15^{a}	$0.75\pm0.15^{a,AC}$	0.75 ± 0.15^{a}	$1.51\pm0.16^{c,E}$	$1.20\pm0.15^{bc,CD}$	$1.38\pm0.15^{bc,BC}$	$1.07\pm0.17^{ab,C}$
VI	0.93 ± 0.15^{ac}	0.90 ± 0.15^{ac}	0.87 ± 0.15^{ac}	$1.62\pm0.15^{b,E}$	$1.30\pm0.17^{ab,D}$	$1.48\pm0.16^{b,BC}$	$0.92\pm0.16^{ac,C}$
VII	0.75 ± 0.15^{a}	0.96 ± 0.15^{ac}	0.99 ± 0.15^{ac}	$1.62\pm0.15^{b,E}$	$1.08\pm0.15^{ac,CD}$	$1.18\pm0.16^{c,A}$	$0.92\pm0.16^{ac,C}$
Positive serum	2.78	2.78	2.63	2.63	2.70	2.63	2.63
(Accepted range)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)	(2.85±0.23)
Negative serum	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75	≤0.75
(Accepted range)	(≤0.75)	(≤0.75)	(≤0.75)	(≤0.75)	(≤0.75)	(≤0.75)	(≤0.75)

Note; Group I; injected with NSS 1 mL via ID Group II; conventional vaccinated FMD vaccine 2 mL via SC, Group III; automatic needle-free vaccinated FMD vaccine 1 mL via ID, Group IV; vaccinated FMD vaccine 0.25 mL via manual ID, Group V; vaccinated FMD vaccine 0.5 mL via manual ID, Group VI; vaccinated FMD vaccine 1 mL via manual ID, and Group VII; vaccinated FMD vaccine 2 mL via manual ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling times ($p<0.05$).

Table 6 The proportion of protective immunity in vaccinated calves against FMDV serotype O (experiment 1).

Group	The proportion of protective level against serotype O expressed as Percentage (%) (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	2.9±7.5	2.9±7.5	2.9±7.5	2.9±7.5	2.9±7.5	2.9±7.5	2.9±7.5
II	2.9±5.3	2.9±5.3	2.9±5.3	11.2±10.5	2.9±5.3	2.9±5.3	2.9±5.3
III	2.9±7.5	2.9±7.5	20±17.9	2.9±7.5	2.9±7.5	2.9±8.4	2.9±8.4
IV	2.9±7.5	2.9±7.5	20±17.9	40±21.9	2.9±7.5	2.9±7.5	2.9±7.5
V	2.9±7.5	2.9±7.5	2.9±7.5	2.9±8.4	2.9±7.5	2.9±7.5	2.9±8.4
VI	2.9±8.4	2.9±8.4	20±17.9	20±17.9	2.9±8.4	2.9±8.4	2.9±8.4
VII	2.9±7.5	2.9±7.5	2.9±7.5	2.9±8.4	2.9±7.5	2.9±7.5	2.9±7.5

Note; Group I; injected with NSS 1 mL via ID Group II; conventional vaccinated FMD vaccine 2 mL via SC, Group III; automatic needle-free vaccinated FMD vaccine 1 mL via ID, Group IV; vaccinated FMD vaccine 0.25 mL via manual ID, Group V; vaccinated FMD vaccine 0.5 mL via manual ID, Group VI; vaccinated FMD vaccine 1 mL via manual ID, and Group VII; vaccinated FMD vaccine 2 mL via manual ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling times ($p<0.05$).

Table 7 The proportion of protective immunity in vaccinated calves against FMDV serotype Asia-1 (experiment 1).

Group	The proportion of protective level against serotype Asia-1 expressed as Percentage (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	0	0	0	0^{A}	0^{C}	0^{A}	0
II	0^{a}	0^{a}	0^{a}	$94\pm7.5^{b,B}$	$62\pm21.7^{b,B}$	$72.3\pm20^{b,B}$	6.2 ± 7.5^{a}
III	0^{a}	0^{a}	0^{a}	$65.1\pm29.6^{b,B}$	13.7 ± 18.3^{C}	18.9 ± 25.8^{A}	0^{a}
IV	0^{ac}	13.4 ± 17^{c}	0^{ac}	$62.5\pm31.6^{bde,C}$	$34.5\pm29.8^{acd,A}$	$87\pm17.5^{e,C}$	13.4 ± 17^{acd}
V	0	0	0	26.4 ± 29.3^{AC}	0^{C}	37.6 ± 29.8^{A}	26.4±29.3
VI	16.6 ± 19.7^{abc}	0^{a}	0^{a}	37.9 ± 29.5^{b}	$55.2\pm33.5^{b,B}$	70.7 ± 30.2^{c}	0^{a}
VII	0^{a}	0^{a}	0^{a}	$84\pm19.5^{b,B}$	$16\pm19.5^{a,C}$	$0^{a,A}$	0^{a}

Note; Group I; injected with NSS 1 mL via ID Group II; conventional vaccinated FMD vaccine 2 mL via SC, Group III; automatic needle-free vaccinated FMD vaccine 1 mL via ID, Group IV; vaccinated FMD vaccine 0.25 mL via manual ID, Group V; vaccinated FMD vaccine 0.5 mL via manual ID, Group VI; vaccinated FMD vaccine 1 mL via manual ID, and Group VII; vaccinated FMD vaccine 2 mL via manual ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling times ($p<0.05$).

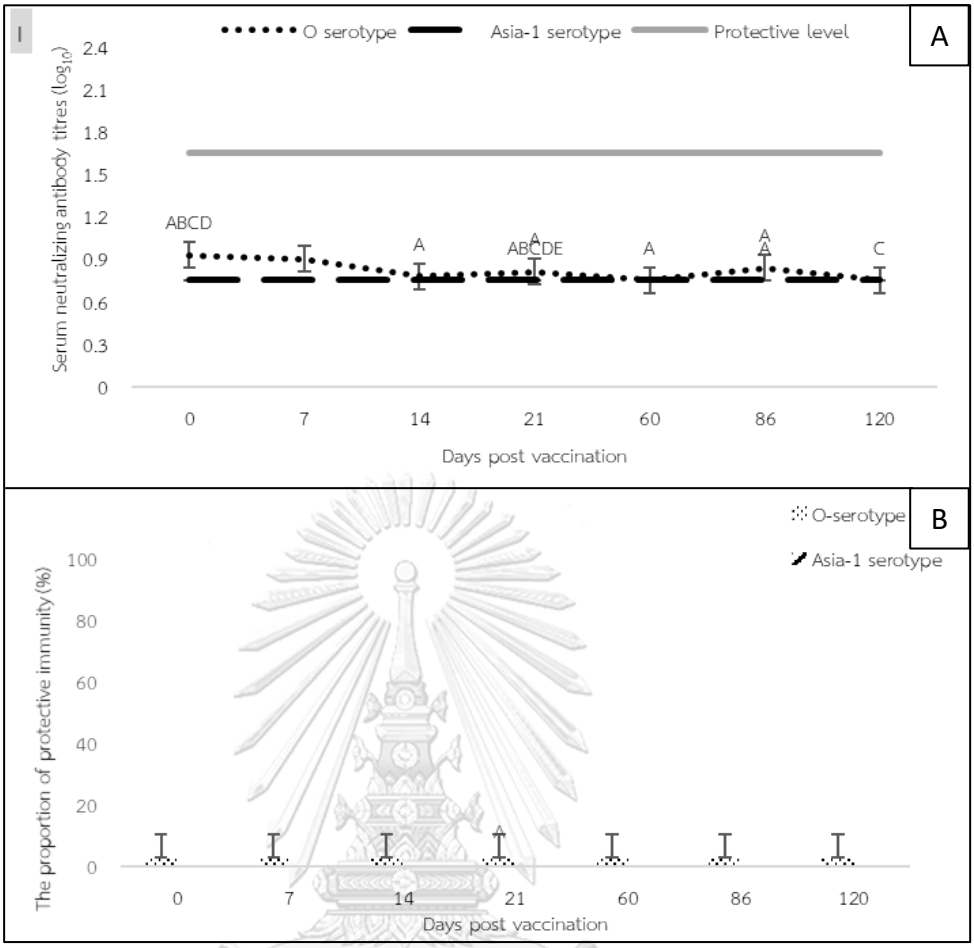

Figure 1 The immune responses against FMDV serotype O and Asia-1 in calves. (Group I; injected with NSS 1 mL via ID)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

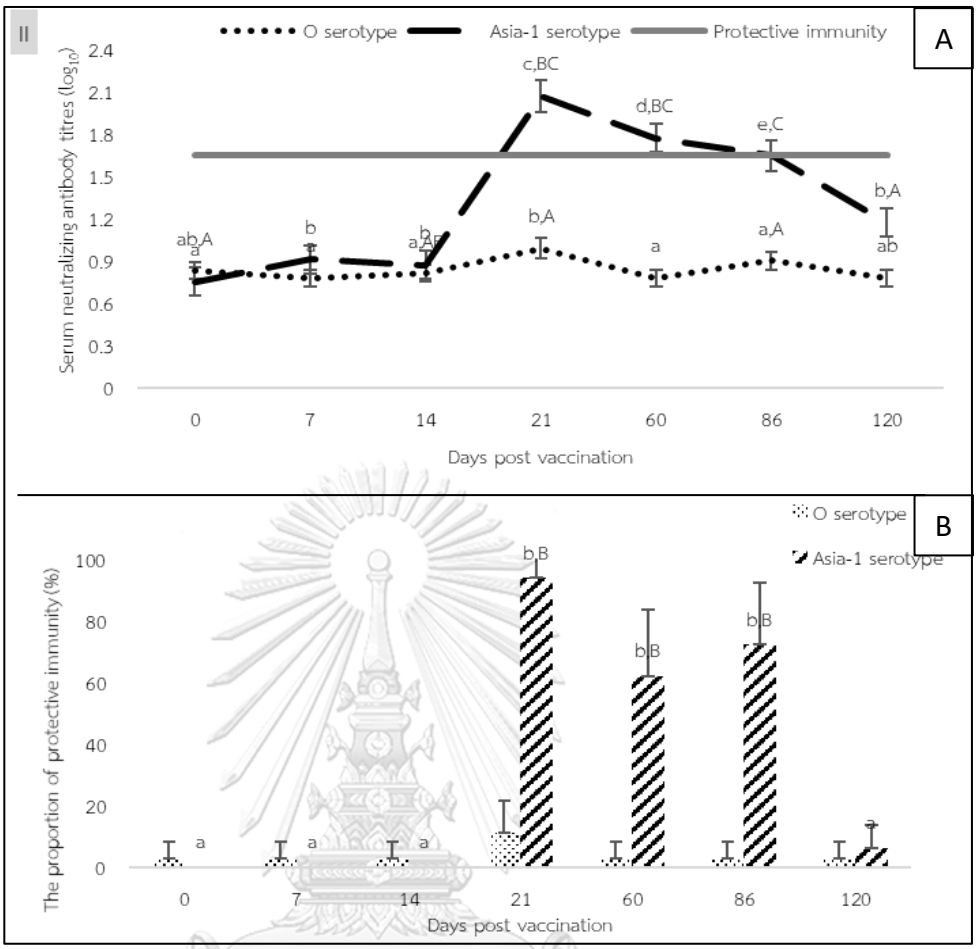

Figure 2 The immune responses against FMDV serotype O and Asia-1 in calves. (Group II; conventional vaccinated FMD vaccine 2 mL via SC)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

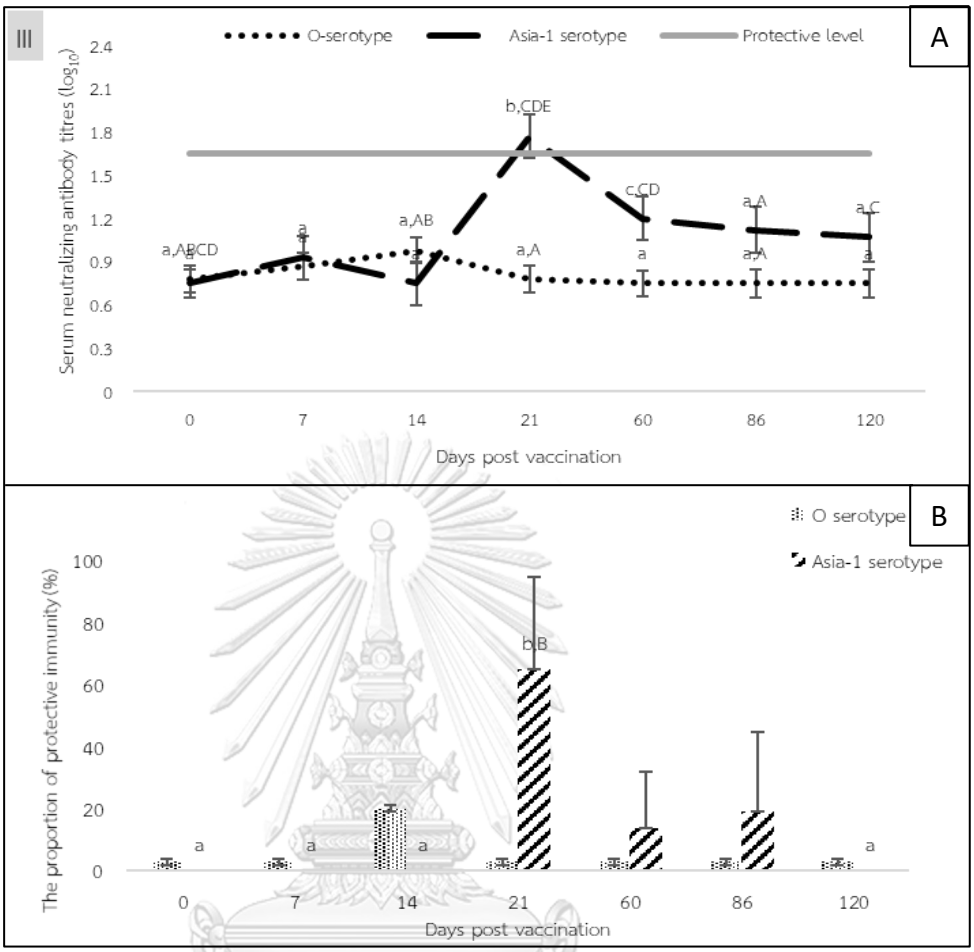

Figure 3 The immune responses against FMDV serotype O and Asia-1 in calves. (Group III; automatic needle-free device with FMD vaccine 1 mL via ID)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

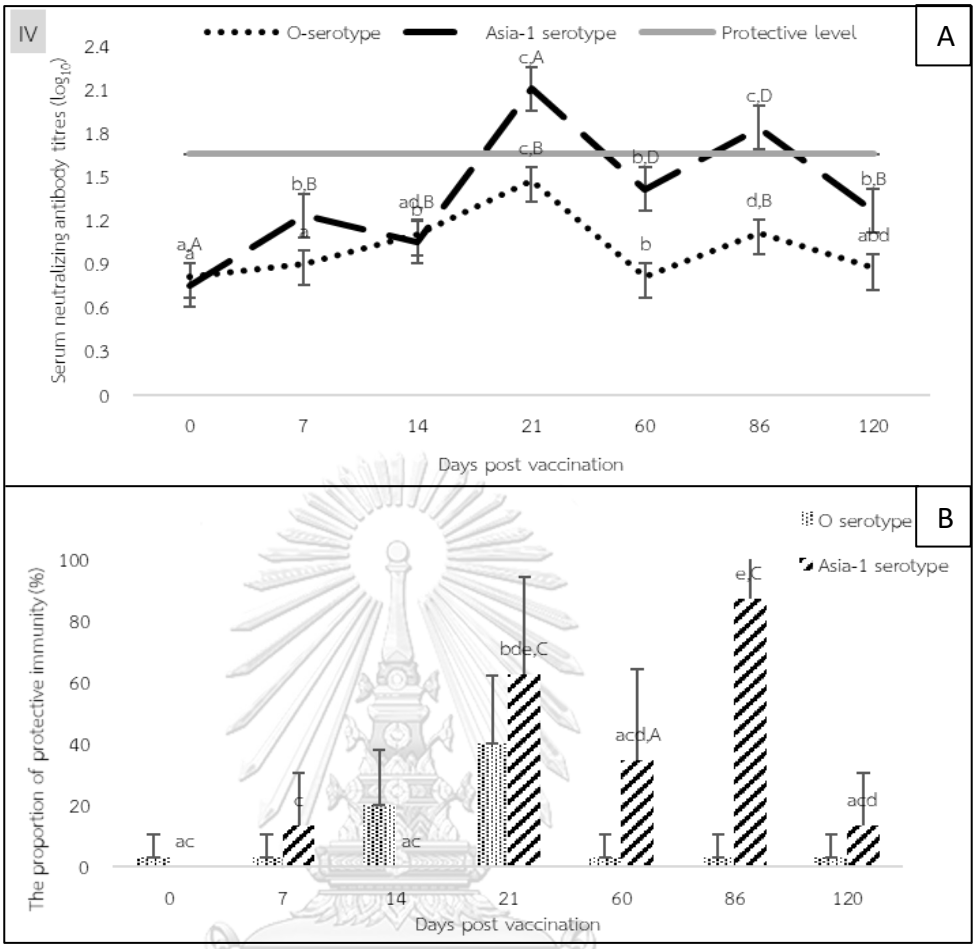

Figure 4 The immune responses against FMDV serotype O and Asia-1 in calves. (Group IV; vaccinated with FMD vaccine 0.25 mL via ID)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

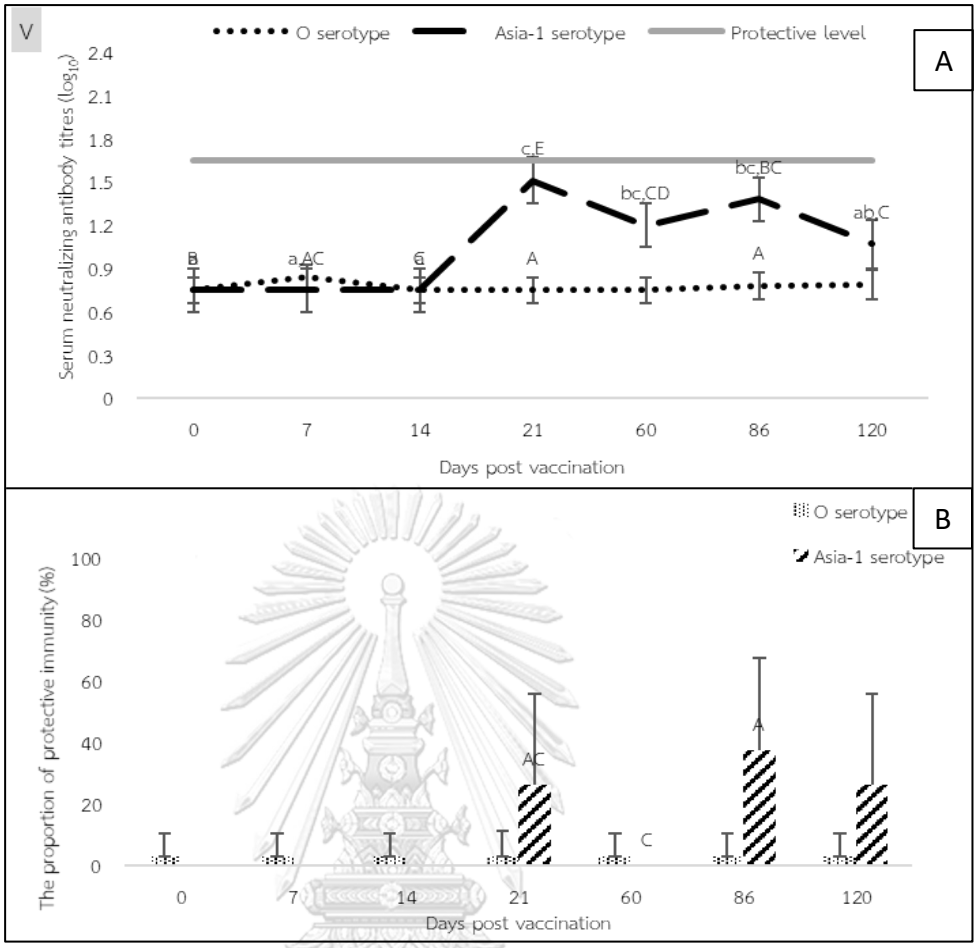

Figure 5 The immune responses against FMDV serotype O and Asia-1 in calves. (Group V; vaccinated with FMD vaccine 0.5 mL via ID)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time (p<0.05).

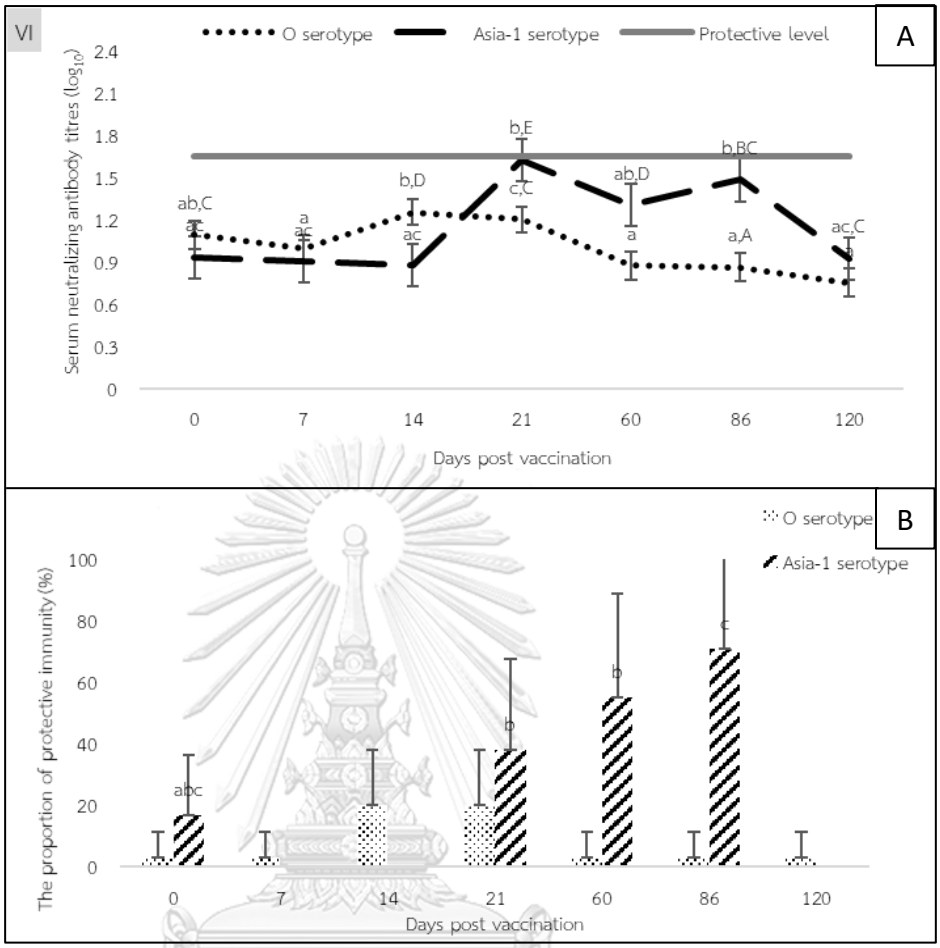

Figure 6 The immune responses against FMDV serotype O and Asia-1 in calves. (Group VI; vaccinated with FMD vaccine 1 mL via ID)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

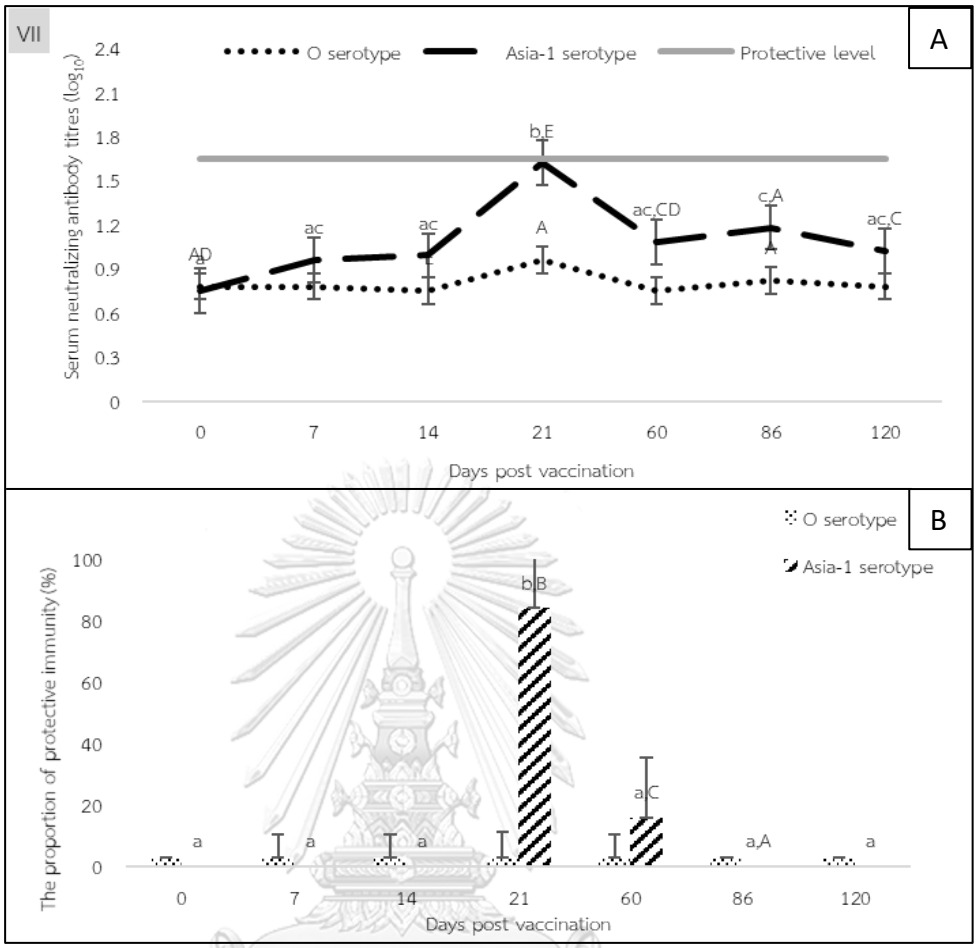

Figure 7 The immune responses against FMDV serotype O and Asia-1 in calves. (Group VII; vaccinated with FMD vaccine 2 mL via ID)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

Table 8 The neutralizing antibody titers (log 10) against FMDV serotype O in heifers (experiment 2).

Group	Neutralizing antibody titer against serotype O expressed as log10 (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	1.13±0.17^{a}	1.92±0.17^{b,AB}	1.78 ±0.17^{bc,A}	1.62±0.17^{c,AB}	1.36±0.17^{a}	1.47±0.17^{ad}	1.20±0.18^{a}
II	0.89±0.17^{a}	1.75±0.17^{b,A}	1.59±0.17^{c,A}	1.40±0.17^{c,A}	1.15±0.18^{ac}	1.20±0.18^{ac}	1.02±0.18^{ac}
III	0.90±0.17^{a}	2.33±0.17^{b,B}	2.19±0.17^{bc,B}	2.03±0.17^{c,B}	1.41±0.17^{d}	1.31±0.19^{d}	1.43±0.18^{d}
IV	0.90±0.17^{a}	1.83±0.17^{b,A}	1.74±0.17^{bc,A}	1.60±0.17^{c,AB}	1.34±0.17^{a}	1.32±0.18^{a}	1.22±0.18^{a}
Positive serum (Accepted range)	2.10 (1.95±0.23)	2.10 (1.95±0.23)	1.73 (1.95±0.23)	1.73 (1.95±0.23)	1.73 (1.95±0.23)	1.80 (1.95±0.23)	1.80 (1.95±0.23)
Negative serum (Accepted range)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)

Note; Group I; conventional vaccinated with FMD vaccine 2 mL via SC, Group II; automatic needle-free vaccinated with FMD vaccine 2 mL via SC, Group III; vaccinated with FMD vaccine 1 mL via manual ID and Group IV; automatic needle-free vaccinated with FMD vaccine 1 mL via ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time (p<0.05).

Table 9 The neutralizing antibody titers (log 10) against FMDV serotype A in heifers (experiment 2).

Group	Neutralizing antibody titer against serotype A expressed as log10 (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	0.75±0.09^{a}	1.26±0.09^{b,A}	1.16 ±0.09^{c,A}	1.01±0.09^{c,A}	0.75±0.09^{a}	0.77±0.09^{a}	0.75±0.10^{a}
II	0.75±0.09^{a}	1.25±0.09^{b,A}	1.10±0.09^{c,A}	1.02±0.09^{c,A}	0.79±0.10^{a}	0.76±0.10^{a}	0.80±0.10^{a}
III	0.75±0.09^{a}	1.69±0.09^{b,B}	1.46±0.09^{bc,B}	1.17±0.09^{c,B}	0.90±0.09^{c}	0.91±0.10^{c}	0.80±0.10^{a}
IV	0.75±0.09^{a}	1.28±0.09^{b,B}	1.14±0.09^{bc,A}	1.01±0.09^{c,A}	0.90±0.09^{a}	0.84±0.10^{a}	0.78±0.10^{a}
Positive serum (Accepted range)	2.10 (1.95±0.23)	2.10 (1.95±0.23)	1.73 (1.95±0.23)	1.73 (1.95±0.23)	1.73 (1.95±0.23)	1.80 (1.95±0.23)	1.80 (1.95±0.23)
Negative serum (Accepted range)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)

Note; Group I; conventional vaccinated with FMD vaccine 2 mL via SC, Group II; automatic needle-free vaccinated with FMD vaccine 2 mL via SC, Group III; vaccinated with FMD vaccine 1 mL via manual ID and Group IV; automatic needle-free vaccinated with FMD vaccine 1 mL via ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time (p<0.05).

Table 10 The neutralizing antibody titers (log 10) against FMDV serotype Asia-1 in heifers (experiment 2).

Group	Neutralizing antibody titer against serotype Asia-1 expressed as log10 (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	0.81±0.16^{a}	1.74±0.16^{b,AB}	1.67 ±0.16^{b,AB}	1.43±0.16^{b,A}	1.10±0.17^{a}	1.44±0.17^{b}	1.05±0.16^{a}
II	0.75±0.16^{a}	1.53±0.16^{b,A}	1.44±0.16^{c,A}	1.19±0.16^{c,A}	1.12±0.17^{abc}	1.17±0.17^{b}	1.06±0.16^{ac}
III	0.75±0.16^{a}	2.03±0.16^{b,B}	1.95±0.16^{bc,B}	1.71±0.16^{c,B}	1.34±0.16^{c}	1.16±0.18^{d}	1.30±0.17^{d}
IV	0.78±0.16^{a}	1.86±0.16^{b,B}	1.79±0.16^{b,B}	1.65±0.16^{b,C}	1.25±0.16^{c}	1.36±0.17^{bc}	1.13±0.17^{c}
Positive serum (Accepted range)	2.10 (1.95±0.23)	2.10 (1.95±0.23)	1.725 (1.95±0.23)	1.725 (1.95±0.23)	1.725 (1.95±0.23)	1.80 (1.95±0.23)	1.80 (1.95±0.23)
Negative serum (Accepted range)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)	≤0.75 (≤0.75)

Note; Group I; conventional vaccinated with FMD vaccine 2 mL via SC, Group II; automatic needle-free vaccinated with FMD vaccine 2 mL via SC, Group III; vaccinated with FMD vaccine 1 mL via manual ID and Group IV; automatic needle-free vaccinated with FMD vaccine 1 mL via ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time (p<0.05).

Table 11 The proportion of protective immunity in which the heifer had NAT above the protective levels against FMDV serotype O (experiment 2)

Group	The proportion of protective level against serotype O expressed as Percentage (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	7.7±10.4^{a}	83.5±16.6^{b}	72.1±23.8^{b}	56.8±29.3^{b}	38.8±29.2^{ab}	38.8±29.2^{ab}	7.8±10.7^{a}
II	1.7±3.2^{a}	84.2±16.6^{b}	54.4±29.8^{ab}	37.2±28.3^{ab}	1.8±3.6^{c}	1.8±3.6^{c}	1.8±3.6^{c}
III	3.3±5.3^{a}	100±0^{b}	100±0^{b}	67.6±25.3^{c}	37.7±27.5^{a}	25.1±23.8^{ad}	11.7±13.9^{d}
IV	1.6±2.9^{a}	92.1±9.5^{b}	92.1±9.5^{b}	57.2±29.5^{ab}	19.9±21.3^{a}	23.9±26^{a}	1.7±3^{a}

Note; Group I; conventional vaccinated with FMD vaccine 2 mL via SC, Group II; automatic needle-free vaccinated with FMD vaccine 2 mL via SC, Group III; vaccinated with FMD vaccine 1 mL via manual ID and Group IV; automatic needle-free vaccinated with FMD vaccine 1 mL via ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time (p<0.05).

Table 12 The proportion of protective immunity in which the heifer had NAT above the protective levels against FMDV serotype A (experiment 2)

Group	The proportion of protective level against serotype A expressed as Percentage (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	0	16.6±14.5	7.2±8.6	7.2±8.6	0	0	0
II	0	6.6±8.3	16.6±14.8	0	0	0	0
III	3.3±0	52.2±24	36.7±22.8	22.7±18	0	0	0
IV	0	15.7±14.1	15.7±14.1	6.5±8	0	0	0

Note; Group I; conventional vaccinated with FMD vaccine 2 mL via SC, Group II; automatic needle-free vaccinated with FMD vaccine 2 mL via SC, Group III; vaccinated with FMD vaccine 1 mL via manual ID and Group IV; automatic needle-free vaccinated with FMD vaccine 1 mL via ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

Table 13 The proportion of protective immunity in which the heifer had NAT above the protective levels against FMDV serotype Asia-1 (experiment 2)

Group	The proportion of protective level against serotype Asia-1 expressed as Percentage (average±SE) on days post vaccination						
	0	7	14	21	60	86	120
I	0^{a}	87.4 ± 13.5^{b}	74.8 ± 20.9^{b}	33.4 ± 22.6^{ab}	0^{a}	33.4 ± 22.6^{ab}	0^{a}
II	0^{a}	58 ± 26.8^{b}	39.3 ± 27.4^{ab}	20.9 ± 20.4^{ab}	9 ± 11.5^{a}	8.8 ± 11.2^{a}	2.2 ± 3.4^{a}
III	0^{a}	89.3 ± 11.5^{b}	79.1 ± 18.5^{bc}	65.2 ± 24.6^{bc}	33.5 ± 23.9^{ac}	11.1 ± 14.1^{a}	15.3 ± 15.8^{a}
IV	0^{a}	88.7 ± 11.7^{b}	67.1 ± 23.2^{bd}	52.8 ± 26.2^{bcde}	22.6 ± 19.8^{ad}	18.3 ± 18.7^{ae}	6 ± 9^{ac}

Note; Group I; conventional vaccinated with FMD vaccine 2 mL via SC, Group II; automatic needle-free vaccinated with FMD vaccine 2 mL via SC, Group III; vaccinated with FMD vaccine 1 mL via manual ID and Group IV; automatic needle-free vaccinated with FMD vaccine 1 mL via ID. The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

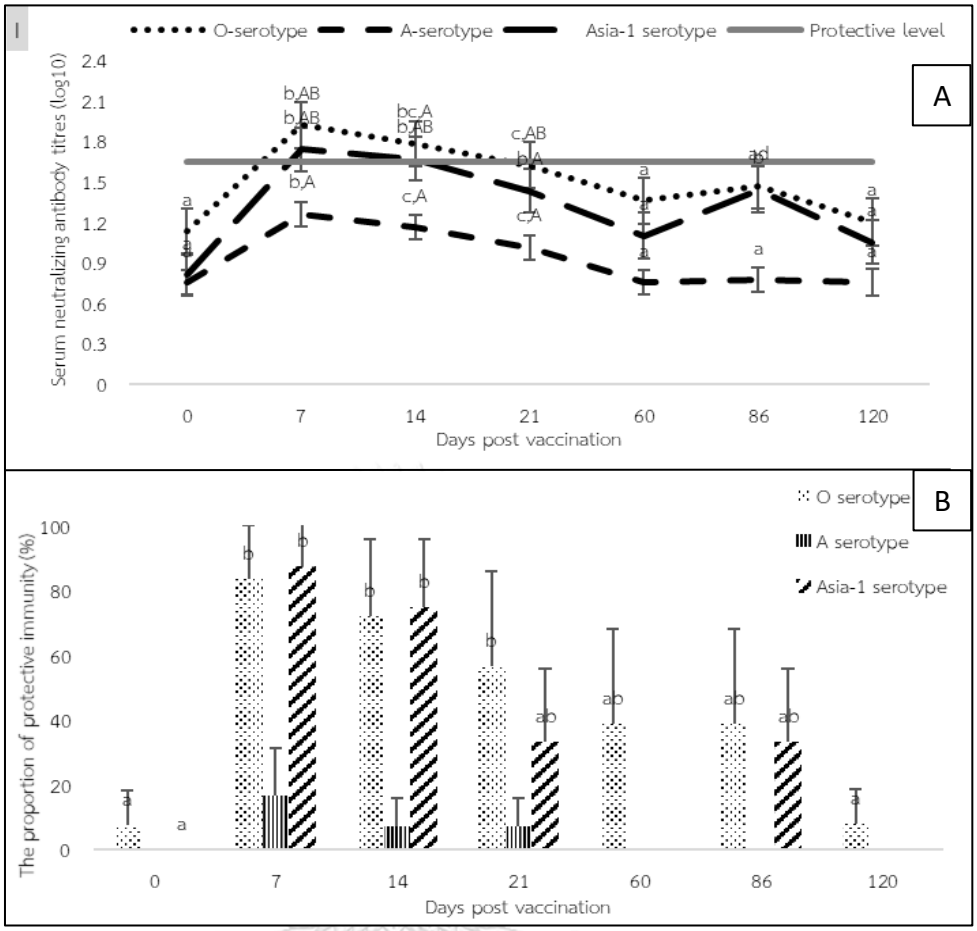

Figure 8 The immune responses against FMDV serotype O, A and Asia-1 in heifers. (Group I; conventional vaccinated with FMD vaccine 2 mL via SC)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O, A and Asia-1
(b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O, A and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time (p<0.05).

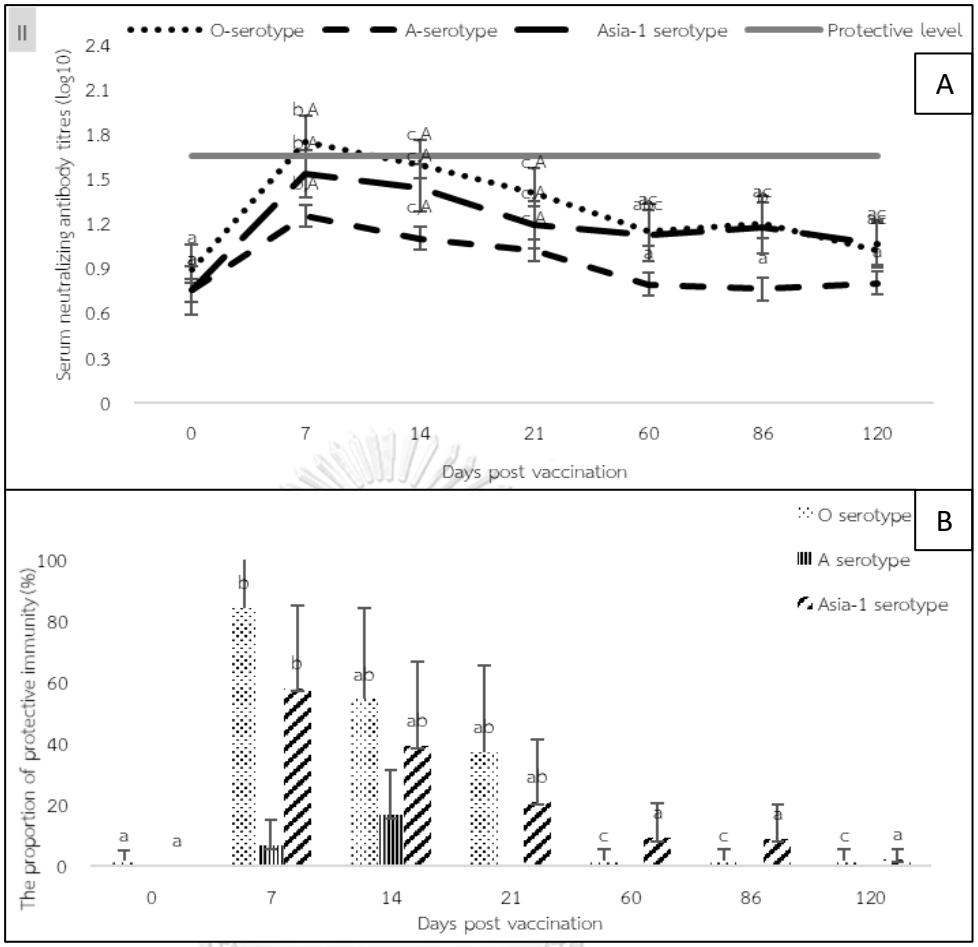

Figure 9 The immune responses against FMDV serotype O, A and Asia-1 in heifers. (Group II; automatic needle-free device with FMD vaccine 2 mL via SC)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O, A and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O, A and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

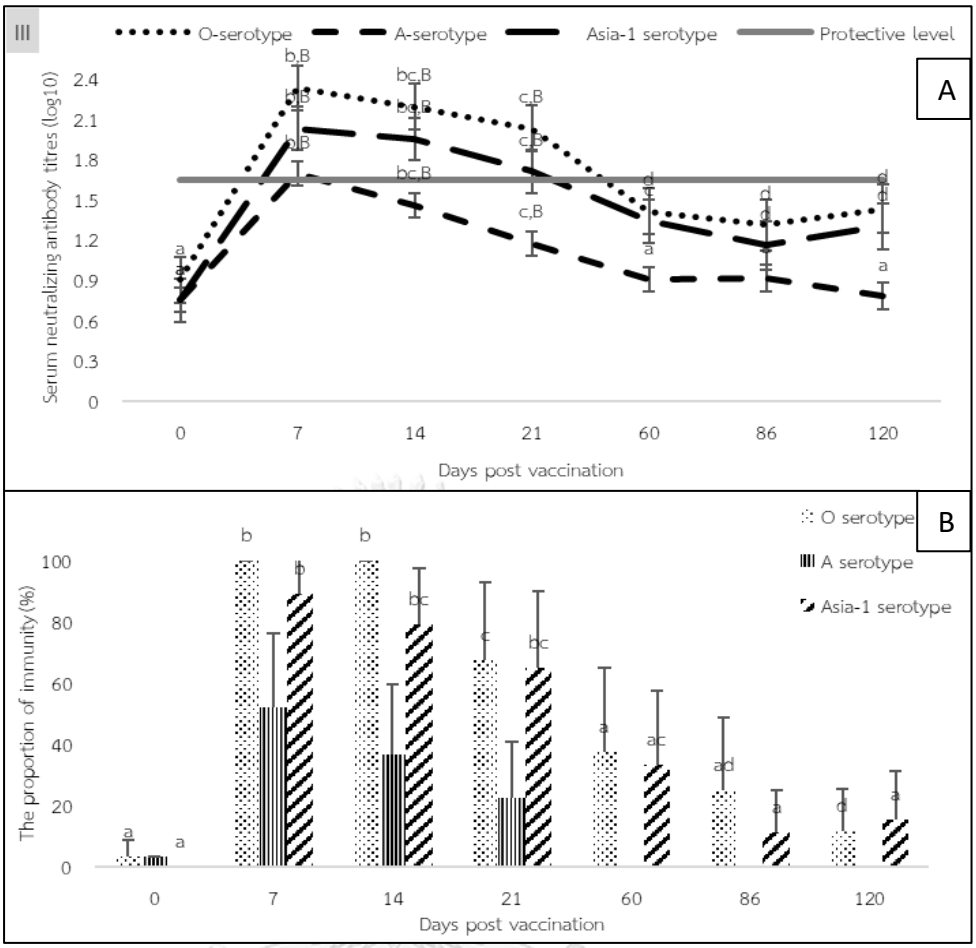

Figure 10 The immune responses against FMDV serotype O, A and Asia-1 in heifers. (Group III; vaccinated with FMD vaccine 1 mL via ID)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O, A and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O, A and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

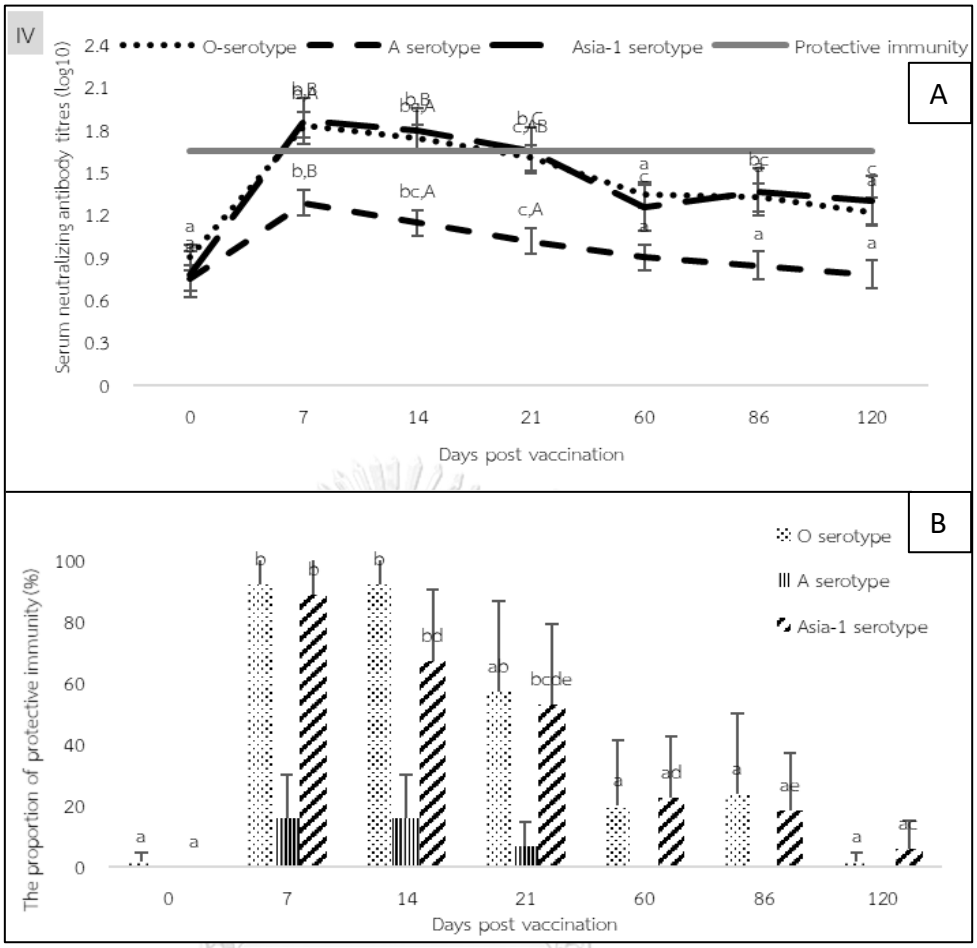

Figure 11 The immune responses against FMDV serotype O, A and Asia-1 in heifers. (Group IV; automatic needle-free device with FMD vaccine 1 mL via ID)

(a) The neutralizing antibody titers (log 10) against FMDV serotype O, A and Asia-1 (b) The proportion of protective immunity in vaccinated animal in which reached the cut-off threshold 1.65 (%) against FMDV serotype O, A and Asia-1

Note; Grey line represents the cut-off threshold of protective immunity (1.65) (OIE,2017). Error bars represent standard errors (SE). The different lowercase letters (a, b, c, d, e) represent significant difference among sampling times within the experimental group and capital letters (A, B, C, D) represent significant difference among experimental groups at the same sampling time ($p<0.05$).

Chapter 5: Discussion

For FMD outbreak in Thailand, one of the important causes is the low protective immunity in animal population. Improvement of alternative vaccine administration to animal by reducing amount of vaccine per dose would increase the number vaccinated animal in population. Intradermal vaccination is not only reduction in usage of vaccine per dose but also induce the immune response faster and longer than SC route (Hunsaker and Perino, 2001). Recently, the automatic needle-free device for vaccination has been studied and revealed that its might be an alternative way of vaccination (Pires et al., 2007; Pandya et al., 2012; Rey et al., 2013; Rey et al., 2015). Reduction of animal restraint, the large number of animals can be vaccinated in order to increase the immunity of population.

In our result, calf had immune response to FMD vaccination in which all vaccinated calf groups (group II- VII) had increased NAT against serotype O and Asia-1 after the boost vaccination. Group IV had the NAT against serotype O and Asia-1 significantly difference from other groups on 21 dpv. The NAT was no significantly difference between group II and III during different sampling times. The proportion of protective immune animals was low for serotype O, while proportion of protective immune animals for serotype Asia-1 increased significantly on 21 dpv, particularly in group II.

All heifer groups had increased significantly immune response. The NAT of all serotypes reached the highest level on 7 dpv. Group III had NAT against serotype O, A and Asia-1 significantly difference from the others. The proportion of protective immunity animal was not significantly difference among group during studied period.

This is the first report of ID administration of trivalent FMDV vaccine produced by DLD. Our study indicated that NAT had increasing immune response in all calves after the second vaccination, particularly in group IV (manual ID 0.25 mL). The immune response of manual ID 0.25 mL may lead to the superior immune response to conventional method. The manual ID 0.25 mL would be benefit in term of dose-sparing (12.5% of standard dose). However, the proper dose ID of FMD vaccine to stimulate an immune response was performed only in the calves, so the study of this topic needs to be further investigated in the heifer and cows.

The ID delivery may be the alternative method for FMD vaccination in dairy cattle. The dermis has an important role in immunity response due to a plenty of dendritic cells which is an antigen-presenting cells in epidermal and dermal layers (Hickling et al., 2011). Samina et al. (1998) stated that the ID vaccination of commercial trivalent FMD vaccine (O, A, and Asia-1) resulted in higher immune response than SC. Another study of ID application in pig indicated that the protection against clinical diseases could be possibly performed with 1/10 antigen dose of the regular dose (Eble et al., 2009). ID is a potential route of vaccine administration in which we postulated the hyperimmune response with a lower dose of FMD vaccine with the adjustment of appropriate antigen.

This study indicated that the immune response of ID dosage 0.25 mL resulted in higher level of average NAT against serotype Asia-1 than ID 0.5, 1 and 2 mL in calves on 21, 60 and 86 dpv. Since the thickness of cattle dermis is 3-5 mm (Itzchak et al., 1992), the anatomical capacity at intradermal tissue is quite narrow in space to inject fluid inside dermal tissue. In this study, the optimal volume for intradermal vaccination in calve is 0.25 mL in order to eliciting efficacious immune response. The study in human trial indicated that the dosage of ID given is usually less than 0.5 mL (Kozier, 2008). The

information of ID in calf is not well published. However, ID injection should be consciously performed. There was a report for over dose, the complication of exceeding dosage was found in the children who received *Bacillus Calmette-Guerin* (BCG) overdose upper limit of the currently recommended ID resulted in the complications such as ulcer (Farries, 1980). For another problem, it might relate to technical problem for ID vaccination in dairy cattle. Due to the skin hardness, animal restraint and personal skill of vaccinator in a small dose of vaccine may be the critical concern in farm practice.

Therefore, the 0.25 mL of FMD vaccine in calves can stimulate the immune response better than the conventional method. In our experiment, we exhibited that the FMD vaccine with minimum dose of 0.25 ID is optimal dose that is able to induce the immune response. In heifer, the results were similar to the study in calf. The vaccinated heifers using manual ID showed the highest immune response against serotype O, A and Asia-1 at 7 dpv. Our results strongly supported that the ID vaccination ia able to stimulate the better immune response with reduced amount of vaccine per dose. The advantage of dose-sparing would be the benefit to the overall costs of vaccine production and vaccine delivery. The increase of vaccinated animal under the current limitation of vaccine distribution in Thailand, using the ID administration is feasible protocol in order to increase the vaccine coverage.

Furthermore, the ID vaccination was investigated in other diseases including bacterial vaccine such as *Salmonella enterica* serotype Dublin in Aitken et al. (1982). The study indicated that ID application can protect against the challenge when compared with SC administration. For the viral vaccine, calves vaccinated with *BHV-1* via the ID in which animal had shown the protection against *BHV-1* challenge (Van Drunen Littel-van den Hurk et al., 1998). The results of the previous studies generally indicated that ID can enhance immune response according to experimental challenged.

The immune response to FMD serotype A had not shown the immune response throughout our experiment. Our study is similar to the study of Elnekave et al. (2016a). Elnekave et al. (2016a) showed the neutralizing antibody titer of A-serotype had lower antibody titer compared to other serotypes.

Our study in calves indicated that the proportions of protective immunity against FMDV serotypes O, and A in vaccinated animals were low (less than 50%). In general, killed FMD vaccine has limited ability to stimulate the immunity and has short duration of protective level (Abdela, 2017). Knight-Jones et al. (2015) reviewed that the short-term duration of killed FMD vaccine protection was interested issue. The calves need to be regularly vaccinated at least five times per year for first year in order to remain the neutralizing antibody titer at the protective immunity level (Elnekave et al., 2016a). The high potency FMD vaccine showed that three months old calf using repeated 3 doses can reach the selected cut-off titer and remains consistency (Elnekave et al., 2016a). Also, the heifers or cows are required three doses with high potency vaccine in which there are able to develop and to maintain adequate antibody levels (Doel, 1999; Elnekave et al., 2016a; Elnekave et al., 2016b) In Thailand, according to our results, we proposed that calf need to be repeated vaccination in order to reach the protective level. The further investigation of this issue should be performed by using FMD vaccine produced by DLD.

The cut-off threshold for determining the protective level is the critical issue. The standardization of the cut-off threshold determination has been established from potency test (Pay and Hingley, 1992). The cut-off titer needs to be standardized difference relevant each serotype from the potency test result. The animal that reached the protective level may be increased.

The immune response of the needle-free device in calves was similar to conventional method. This findings are agreed with the other studies in cattle of Rey et al. (2015). The previous researches in cattle supported that the needle-free injection resulted in comparable immune response to needle syringe injection (Hollis et al., 2005; Pires et al., 2007; Pandya et al., 2012; Rey et al., 2015). Another study shown that the antigen can be reduced to 1/16 of a regular dose and can protect FMD in cattle using the intradermal vaccination with needle-free device (Pandya et al., 2012). The study of Pandya et al. (2012) was used dosage of injected 0.5 mL by the needle-free device via ID. Thus, the needle-free system can be alternative way for FMD vaccination. In this study, we proved that the Pulse 250® Needle-free systems model (Pulse®,USA), is appropriate for FMD vaccine administration for dairy cattle. However, the appropriate dosage for this device need to be further study.

Moreover, the benefits of the device reduced lateral transmission of disease (Reinbold et al., 2010) such as bovine leukemia virus that induced leukemia/lymphoma mortality (Hopkins and DiGiacomo, 1997; Weese and Jack, 2008; Erskine et al., 2012), blood-borne infection (Otake et al., 2002) and reduced infection due to a repeated used dirty needle (Skilton and Thompson, 2005).

This study indicated that the automatic needle-free device via intradermal can substituted with the standard method. This device can be performed with rapid multi-doses vaccinator. It is convenience, less labor cost and less animal stress during restraint. The device can stimulate the antibody responses comparable to conventional needle vaccination. This procedure can eliminate the hidden danger of the injection needle, gain the benefits of the reduction of societal costs in which difficult to quantify in the financial

term, for example, the safety of workers which needlestick injuries and vaccination wastage such as disposable-syringe.

However, the current model (Pulse 250®) has a limitation of dose adjustment (1, 2 and 5 mL). The further research and development, the device need to be modified or adapted to be more flexible for dose adjustment for less volume such as 0.1, 0.25 and 0.5 mL.

In heifer group IV (automatic needle-free ID 1 mL), the neutralizing antibody titer against FMDV serotype O, A and Asia 1 were not different from group I (conventional SC 2 mL). This result supported the calf study in which the efficacy of the needle-free device via ID can also substitute to standard needle syringe SC. Our study is the first report that showed the efficacy of ID delivery by the needle-free device in FMD vaccination in dairy cattle in Thailand. The needle-free device can be the alternative way of FMD vaccination in the dairy cattle. The result showed that the average level of serum neutralization test in the needle-free device group had not difference immune response compared to the conventional group. In our study, using needle-free device for 1 mL of FMD vaccine can elicit levels of neutralizing antibodies after vaccination which showed agreement with the study of Pandya et al. (2012).

The proportion of immune protective vaccinated animals were high during 1-2 months after vaccination. This proportion had slightly declined after two months. In general, the duration of immune protection is last for 4-6 months (Doel, 2003). Abdela (2017) stated that the regular FMD vaccine with $\geq 3PD_{50}$ can induce 2/3 of vaccinated animal to reach the protective level. However, this protection could be last for 3-4 months after the vaccination.

The FMD vaccine provided the limited short-termed disease protection. The regular in every four months is essential for maintaining the

protective immunity (Knight-Jones et al., 2016). The protective immunity of Thai DLD vaccination program is needed to be further studied in order to evaluate the duration of protective immunity in vaccinated animals.

In heifer, the level of protective immunity against FMDV serotypes O, A and Asia-1 showed high proportion of vaccinated animals. These animals reached the protective immunity level because they had been previously vaccinated at least two times. The memory B cells in their immune system is rapidly response in which called the anamnestic response. At the beginning, the proportion of vaccinated heifers showed low level of protection. After our first vaccination, the proportion of vaccinated animals can reach the protective immunity more than 70%.

In our study herd, we suggested that all animal should be regularly vaccinated every three months. However, the calves should be initially vaccinated at four months old with a month consecutively booster dose. This vaccination program would be benefit to increase immunity response and to maintain herd protective immunity.

In conclusion, the intradermal needle-free device revealed the good immune response to FMD vaccination in both calf and heifer. The optimal dose for an intradermal route of FMD vaccine is 0.25 mL in which suitable to stimulate an immune response. However, the duration of protective immunity after vaccination in both calf and heifer were short term period. The vaccination program should be reconsidered. Moreover, the quality of vaccine should be improved for inducing a long period of immunity protection. The further study in field experiments with an appropriated dose of FMD vaccine via from DLD should be examined.

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