On-farm trial of modified live Classical swine fever vaccine induced neutralizing antibody titers: Comparison of intradermal and intramuscular vaccination methods

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Abstract

The aim was to investigate on-farm application of an alternative Classical swine fever (CSF) vaccination, whether intra-dermal (ID) vaccination is suitable as an alternative for traditional intramuscular (IM) route in an organic pig farm. We compared antibody titers by using neutralizing peroxidase-linked monolayer assay (NPLA) between pigs vaccinated with lived CSF vaccine containing a normal or 5-fold reduced dose. The vaccine was government produced (Thailand), containing $10^2$ PD$_{50}$ (50% protective dose) of modified live Lapinized Chinese-strain CSF virus. A total of 18 nursery pigs were pair matched (according to litter, weight, and sex) and divided into 2 groups. Amount of 0.2 ml or 1 ml full-dose of vaccine was administered ID or IM, respectively. As compared with the 1ml IM vaccinated pigs, the pigs vaccinated ID with 0.2ml of the same vaccine had comparable antibody titers to IM group. Geometric mean titers (log10) at day 0, 14, 21, and 28 days post-vaccination of ID group were 5.44, 7.41, 11.76, and 38.05, and GMT of IM group were 4.67, 8.64, 12.70, and 32.00, respectively. We conclude that the ID route can be used as an alternative for IM application of CSF vaccine.

Keywords:  CSF, ID vaccination, neutralizing antibody, NPLA
เปรียบเทียบแอนติบอดีจากการฉีดวัคซีนอีฮัวตัวสุกรซื้อเป็นแบบเข้าล้มและเข้าในหนัง โดยทดสอบในฟอร์มสุกร

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บทคัดย่อ

วัคキュลินส์เพื่อทดสอบการประยุกต์ใช้การฉีดวัคซีนแบบในหนัง (ID) ใช้วัคซีนเป็นชนิด 0.2 มล. ให้ผ่านในการกระตุ้นแอนติบอดีได้มากเทียบเท่าการฉีดเข้ากล้าม (IM) ใช้ 1 มล. แบบดังกล่าว โดยทดลองในชีวๆสุราโยธาศาสตร์ คณะและหน่วยงานวัคซีน จึงตัดตัวสุกรเพื่อความสามารถระดับแอนติบอดีโดยวิธี NPLA วัคซีนทีวัคซีนสุกรสุกรเป็นชนิดซึ่งเป็นสายพันธุ์ Chinese เพื่อจำแนกในการ ในชีวิตต่างประเทศ ปริมาณของผลหัวขึ้นของไวรัสที่มี 10^2 PD₅₀ (50% protective dose) สองหนึ่ง มล. ชุดที่หนึ่งได้ที่ 18 วัน แบ่ง 2 กลุ่มโดยกลุ่มละ 2 กลุ่ม และวัคซีนได้ผลสูงที่สุด ระดับของ NPLA (GMT: log10) ในวันที่ 0, 14, 21 และ 28 หลังฉีดวัคซีน (dpv) ของ ID คือ 5.44, 7.41, 11.76 และ 38.05 ของ IM คือ 4.67, 8.64, 12.70 และ 32.00 ตามลำดับ ระดับ GMT ของที่สูงที่สุด (p<0.05) ในขณะที่ระดับในกลุ่มที่ 21 dpv ผล 28 dpv สรุปได้ว่า ID สามารถใช้เป็นทางเลือกแทน IM ในการฉีดวัคซีนอีฮัวตัวสุกรแบบซื้อเป็น

คำสำคัญ: อีฮัวตัวสุกร ผิวติดบอดี NPLA
Introduction

Classical swine fever (CSF) is notorious as a severe, highly contagious and economically significant viral disease of pigs. Only domestic pigs, wild boar and feral pigs are susceptible to CSF virus (Moennig and Greiser-Wilke, 2008). CSF is classified by the Office International des Épizooties (OIE) as a list A disease. It is present in Europe, Central and South America, and Asia, but absent from Australia, New Zealand, and North America (Blome et al., 2010). Re-emerging of CSF can be devastating. According to OIE (2009), in 1997-1998, an outbreak in the Netherlands involved more than 400 herds and cost $2.3 billion to eradicate (Martínez-López et al., 2011). The United Kingdom experienced an epidemic in 2000, and minor outbreaks were reported in Romania, Slovakia, Spain and Germany in 2001 (Karsten and Kieter, 2005; Boklund et al., 2009; Blome et al., 2010). Major reported CSF in Thailand in 2011, 2012, 2013, and 2014 were 4, 8, 22, and 8 outbreaks, respectively (Kamakawa et al., 2006; Kortekaas et al., 2011) (Joint FAO/OIE Workshop on Swine Disease Control in Asia Beijing, PR China, 18-20 November 2014).

Currently, CSF disease continues to occur in many countries across the world and especially in Asia. In countries where CSF is endemic, vaccines may be used to protect animals from clinical disease. Vaccination can reduce prevalence of infection during an eradication program (Kortekaas et al., 2011; Dürr et al., 2013; Zheng et al., 2013). Both modified live and subunit (marker) vaccines are manufactured, although availability varies with the countries. Traditional CSF vaccines are of three types; 1) inactivated CSF virus, 2) inactivated bovine diarrhea virus, and 3) live-attenuated CSF vaccines. Due to poor efficacy of the first two vaccines (Koenig et al., 2007; Kortekaas et al., 2011), only live-attenuated vaccine is now routinely used. A modern type of inactivated vaccine is developed by genetic engineering methods (Dong and Chen, 2007). Until now, only subunit marker vaccine based on recombinant Baculovirus-expressed E2 is commercially available (Bouma et al., 1999). Glycoprotein E2 subunit vaccine was capable of inducing protective immunity as well as distinctive antibody (ie., marker vaccine) (de Smit et al., 2001; Moormann et al., 2000; Sung et al., 2011; Renson et al., 2014; Lévai et al., 2015). However, E2 subunit vaccine could not prevent horizontal or vertical transmission (Moormann et al., 2000; Blome et al., 2014).

There are 3 strains of CSF modified live vaccines (MLV), Chinese or C strain, Japanese guinea-exaltation-negative or GPE-, and French Thiverval strains (Qiu et al., 2006). The MLV can induce a rapid and solid protection against a lethal challenge with CSF virus (Moennig and Becher, 2015). Blanket vaccination is routinely practiced in Thailand during CSF outbreaks (Suradhat et al., 2007). All registered vaccines are administered intramuscularly with 1 or 2 ml per dose. Giving reduced dosage of the vaccine while still activating sufficient immune response, vaccination cost can decrease tremendously. Reduction of vaccine doses might be accomplished by intra-dermal (ID) injection. Skin is populated with Dendritic and Langerhan cells, types of local Lymphocytes, which are efficient and potent antigen-presenting cells for induction of protective immunity, and can result in antigen dose-sparing (Weniger and Glenn, 2013; Tozuka et al. 2016). Many studies have shown that ID vaccination had greater or comparable immune responses such as hepatitis B (Chau et al., 2004; Barraclough et al., 2009; Roukens...
et al., 2010; Bunupuradah et al., 2011), rabies (Ambrozaitis et al., 2006; Roukens et al., 2008; Shiota et al., 2008; Tanisaro et al., 2010; Yanagisawa et al., 2012; Lau and Hohl, 2013; Wongsaroj et al., 2013), Foot and mouth (Eblé et al., 2009), Pseudorabies (van Rooij et al., 1998), Porcine reproductive and respiratory syndrome (Martelli et al., 2007), and influenza vaccines (Auewarakul et al., 2007; Chuaychoo et al., 2010; Esposito et al., 2011; Ansaldi et al., 2012; Basileo et al., 2014; Arakane et al., 2015). It is also less painful than intramuscular injection and causes minimal tissue trauma. The ID injecting tool is easy to use (Jarrahian et al., 2012), and recently is becoming popularity especially in Thailand. The ID needle-free is convenient and injecting process is very quick. In our study, we manipulate ID manually in order to be ascertain that every pig get the vaccine antigen.

**Materials and methods**

**Animals and experimental design**

The trial was performed on an organic pig farm using 18 conventionally reared 6-weeks old piglets born of sows vaccinated with CSF vaccine once a year. The farm was a small-holder with 3-5 sows. The pigs were serologically negative to Porcine Circovirus, Porcine Reproductive and Respiratory Disease, Pseudorabies virus, and *Mycoplasma hyopneumoniae*. The only vaccine used on this farm is CSF. The pigs in each group thus represent youngsters of the same sow in order to reduce variation in their antibody titers. At the beginning, the pigs were dewormed, ear tagged, weighted, and bled. The pigs were pair wise divided into ID and IM groups, according to their weight, sex, and litter. At Day 0, pigs in ID group were injected intra-dermally (ID) at the back of their ears with 0.2 ml of CSF vaccine by using tuberculin syringes. Pigs in IM groups were vaccinated intramuscularly at the neck muscle with 1 ml dose.

The Lapinized Chinese-strain modified-lived vaccine used in this experiment is produced by the Department of Livestock Development, Thailand. It is a modified live vaccine containing $10^2 \text{PD}_{50}$ (50% protective dose) of the virus. The manufacturers recommended dose of the vaccine is one milliliter injecting intra-muscularly.

Tuberculin syringe connected with a $\frac{1}{2}$ inch 24 gauge needle was used to manually inject the antigen of CSF vaccine at the back of pig’s ears. If the procedure was done correctly, a vesicle of 0.3-0.5 centimeter diameter would appear at the skin-site of inoculation. After vaccination, the pigs were inspected daily for clinical signs and rectal temperature. The location at which the vaccination was given was examined at 24, 48, and 72 hours for local hypersensitivity. Ten milliliters of blood samples were collected at 0, 14, 21, and 28 days post-vaccination (dpv). Serum samples were transferred to the tubes and centrifuged, collected the clear fluid portion, and stored at -20 °C until used.

**Neutralizing Peroxidase-Linked Assay (NPLA) and statistical analysis**

Neutralization test was performed in cell cultures using a constant-virus/varying-serum method. As CSF virus is non-cytopathic, any non-neutralized virus must be detected, after multiplication, by an indicator system. Neutralizing antibody titers (SN-titers) against CSF virus in serum samples were measured using the NPLA as described previously (OIE manual, 2008). End-point titers were calculated as the reciprocal of the final
serum dilution that neutralized 100 TCID₅₀ of CSF viruses in 50% of the wells.

The NPLA was carried out in flat-bottomed microtiter plates. Sera were first inactivated for 30 minutes at 56 °C. Dilutions of serum in growth medium (Eagle’s MEM, 5% fetal bovine serum and antibiotics) were dispensed in 50 μl volumes into duplicate wells of a flat bottom microtiter plate. The fetal bovine serum must be free from both Bovine viral diarrhea virus and antibodies to it. A third well was included for each sample. This well contains serum only and was used as a serum control (for cytotoxicity and/or nonspecific staining). The amount of 50 μl of virus suspension, diluted in growth medium to contain approximately 100 TCID₅₀, was added to the wells, and mixed on a microplate shaker for 20 seconds. The plates were incubated in a CO₂ incubator for 1 hour at 37 °C. After mixing, 50 μl of growth medium containing 2 x 10⁵ cells/ml were added. The cells were allowed to grow at 37 °C in 5% CO₂ to become confluent, usually within 3-4 days. The growth medium was discarded and the plates were rinsed once in 0.15 M NaCl. The plates were then drained by blotting on a paper towel. The plates were fixed with 80% acetone and incubated at 70-80 °C for 1 hour. Fifty μl of a hyperimmune porcine CSF antiserum, diluted in 0.5 M NaCl (containing 1% Tween 80 + 0.1% sodium azide, pH 7.6) was added to each well. The plates were then incubated at 37 °C for 15 minutes. The working dilution of the antiserum was determined by prior titration: and serum with an NPLA titer of 1/30,000 was used as recommended. The plates were washed five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6. Then 50 μl of an anti-porcine or anti-murine (as appropriate) IgG-HRPO conjugate (diluted to its working concentration in 0.5 M NaCl with 1% Tween 80, pH 7.6) was added followed by incubation for 10 minutes at 37 °C. The plates were then washed five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6). Chromogen-substrate solution was then added (50 μl to each well) and allowed to stand for 15-30 minutes at room temperature for staining. The test results were read visually. Infected cell sheets were completely or partially stained reddish brown. The cytoplasm of infected cells stained dark red. The monolayers were examined by low-power microscopy to determine the end-point of the titration. The following controls were included in the test: cell control, positive serum and back titration of test virus. The back-titration confirmed that virus has been used at a concentration of between 30 and 300 TCID₅₀/μl.

Differences in SN-titers post vaccination between ID and IM vaccinated groups were statistically analysed using the non-parametric Kruskal Wallis test (SPSS version 14.0). A non-parametric permutation test was used for pair-wise comparison between groups if the Kruskal-Wallis test gave a significant result. All significance levels were set at p<0.05.

**Results**

**Clinical signs and rectal temperature**

After vaccination, no systemic reactions were observed in any of the vaccinated pigs ie., no fever, no sign of depression, the pigs were alert and had good appetite (Table 1). In the IM vaccinated pigs, no local reactions could be seen at the site of injection. In the ID vaccinated pigs, the vesicle disappeared within 24 hours. Only red colored patch at the location of vaccination could be observed with approximately a one centimeter diameter which disappeared within 2 days.
Serological responses

On the day of vaccination (Day 0), all pigs had serum neutralizing antibody (SN) titers ranging from 1:2 to 1:16 (Table 1). After vaccination the SN titers of most pigs increased in both IM and ID groups. Some pigs had same or decreased titers. When SN titers were converted into geometric mean titers (GMT; Log10), there was no significant difference of GMTs between ID and IM vaccinated pigs at 0, 14, 21, and 28 days post-vaccination (dpv) (p>0.05) (Table 2). The GMTs of the IM vaccinated pigs were generally higher than those of the ID vaccinated pigs. Pigs vaccinated by ID and IM methods showed increased GMTs at 14 dpv which increased significantly at 21 dpv (Table 3).

Antibody titers of pigs vaccinated IM at the recommended dose rate of one ml were comparable to titers of the pigs vaccinated ID with 0.2ml of the same vaccine. Geometric mean titers (log10) at day 0, 14, 21, and 28 days post-vaccination of ID group were 0.67, 0.85, 1.10, and 1.51 and of IM group were 0.77, 0.87, 1.08, and 1.58, respectively. We conclude that the ID route can be used as an alternative for IM application of CSF vaccine.

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Sex</th>
<th>Weight (Kg)</th>
<th>Vaccination Route of vaccination</th>
<th>Rectal Temperature (°C)</th>
<th>Serum neutralizing antibody titer SN antibody titers at days post vaccination (dpv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>5</td>
<td>ID</td>
<td>38.6</td>
<td>1:2 1:2 1:8 1:64</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>12</td>
<td>ID</td>
<td>39.4</td>
<td>1:4 1:32 1:64 1:128</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>7</td>
<td>ID</td>
<td>39.0</td>
<td>1:4 1:4 1:8 ND</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>9</td>
<td>ID</td>
<td>39.4</td>
<td>1:8 1:16 ND</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>9</td>
<td>ID</td>
<td>38.8</td>
<td>1:8 1:4 1:8 ND</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>7</td>
<td>ID</td>
<td>39.4</td>
<td>1:4 1:4 1:4 ND</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>5</td>
<td>ID</td>
<td>39.4</td>
<td>1:8 1:4 1:8 1:16</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>7</td>
<td>ID</td>
<td>39.0</td>
<td>1:16 1:16 1:16 1:16</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>9</td>
<td>ID</td>
<td>39.0</td>
<td>1:8 1:16 1:16 ND</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>5</td>
<td>IM</td>
<td>38.3</td>
<td>1:2 1:4 1:4 1:32</td>
</tr>
<tr>
<td>11</td>
<td>Female</td>
<td>10</td>
<td>IM</td>
<td>39.5</td>
<td>1:8 1:16 1:32 ND</td>
</tr>
<tr>
<td>12</td>
<td>Male</td>
<td>8</td>
<td>IM</td>
<td>38.6</td>
<td>1:8 1:8 1:16 1:32</td>
</tr>
<tr>
<td>13</td>
<td>Female</td>
<td>9</td>
<td>IM</td>
<td>39.2</td>
<td>1:4 1:4 1:4 ND</td>
</tr>
<tr>
<td>14</td>
<td>Female</td>
<td>9</td>
<td>IM</td>
<td>39.5</td>
<td>1:8 1:16 1:16 ND</td>
</tr>
<tr>
<td>15</td>
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<td>7</td>
<td>IM</td>
<td>39.7</td>
<td>1:8 1:32 1:128 1:128</td>
</tr>
<tr>
<td>16</td>
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<td>7</td>
<td>IM</td>
<td>38.0</td>
<td>1:2 1:4 1:8 1:64</td>
</tr>
<tr>
<td>17</td>
<td>Male</td>
<td>6</td>
<td>IM</td>
<td>38.8</td>
<td>1:8 1:4 1:4 1:4</td>
</tr>
<tr>
<td>18</td>
<td>Male</td>
<td>12</td>
<td>IM</td>
<td>39.8</td>
<td>1:2 1:16 1:16 ND</td>
</tr>
</tbody>
</table>

ND = not determined, no sample due to the pigs were sold out
ID = intradermal
IM = intramuscular
Table 2. Comparison of geometric mean titers (GMT; log10) against Classical swine fever virus between ID and IM groups

<table>
<thead>
<tr>
<th>Days post-vaccination</th>
<th>Intra-dermal vaccination</th>
<th>SEM</th>
<th>Intra-muscular vaccination</th>
<th>SEM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>5.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.40</td>
<td>4.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98</td>
<td>0.71</td>
</tr>
<tr>
<td>Day 14</td>
<td>7.41&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3.31</td>
<td>8.64&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3.15</td>
<td>0.69</td>
</tr>
<tr>
<td>Day 21</td>
<td>11.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.12</td>
<td>12.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.18</td>
<td>0.91</td>
</tr>
<tr>
<td>Day 28</td>
<td>38.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>62.53</td>
<td>32.00&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.21</td>
<td>0.69</td>
</tr>
<tr>
<td>Mean</td>
<td>9.57</td>
<td>4.58</td>
<td>9.93</td>
<td>5.51</td>
<td>0.83</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup> Letters indicate significant (p<0.05)

Table 3. Geometric mean titers (GMT; Log10) against CSF virus of all pigs vaccinated intradermal and intramuscular routes before and after vaccination

<table>
<thead>
<tr>
<th>Day post-vaccination</th>
<th>Geometric mean titers (GMT)</th>
<th>Standard error of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>5.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.84</td>
</tr>
<tr>
<td>Day 14</td>
<td>8.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.22</td>
</tr>
<tr>
<td>Day 21</td>
<td>12.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.13</td>
</tr>
<tr>
<td>Day 28</td>
<td>34.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.59</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Letters indicate significant (p<0.05)
Discussion

At the start of vaccination (Day 0), all pig were approximately 42-46 days old. Majority of the pigs had SN titers of 1:2 to 1:4 which is the proper time for CSF vaccination. Maternal antibodies at these levels will not impede immune respond to vaccination. In previous study, maternal SN titers of greater than 1:64 inhibited efficacy of vaccination (Suradhat and Damrongwatanapokin, 2003). It was relatively surprising that pigs with titers of 1:8 at Day 0, their SN titers slightly dropped at 14 dpv. This phenomenon occurred in one IM pig, and 2 ID vaccinated pigs. However, SN titers of these 2 ID vaccinated pigs then re-increased above their baseline on 21 dpv afterward. The inclination of antibody responses in these pigs with existing MDA seemed to be slow. Unlike the vaccination in MDA-free or naïve pigs which protection and antibody production could be detected as early as 7 dpv (Lévai et al., 2015). There were 1 IM and 2 ID vaccinated pigs still had the same levels of SN titers from Day 0 to 28 dpv. Pigs in ID groups with SN titers of 1:4 and 1:16 at Day 0, had the same titers after vaccination. Titer of one of the low titer-IM pig remained 1:4 until 28 dpv. These vaccine non-responders were found in both IM and ID pigs, and accounted for 16.67% (3/18 pigs). This finding is different from previous study that stated that ID vaccination route could increase immune response in low-responders (Sugimura et al., 2008; Basileo et al., 2014; Chan et al., 2014). This was not true in our experiment.

A recent work proposed that postnatal persistent CSF infected pigs could occur (Munoz-Gonzalez et al., 2015). Persistently infected pigs will not exhibit antibody response to vaccination while at the same time have virus replication. However, antibody of the pig was not absent in our experiment. It is, therefore, just a matter of time or MDA interferes antibody respond to vaccination. In general recommendation, modified live CSF vaccine is given to young or nursery pigs only one shot. In some vaccinated pigs, although SN antibody was absent, the role of cell mediated immunity had been implicated as evident by clinical protection after challenge (Graham et al., 2010; Graham et al., 2012).

From our results, there must be an explanation on how can ID route induce sufficient immune responses even with a 1/5 fold reduced dose. There must be a special way of immune induction. Antigen presenting cells (APC) are highly populated at the skin such as Dendritic and Langerhans cells. These cells are specialized in antigen presentation. An enhancement of cytotoxic T lymphocytes (CTL), helper T cells or humoral responses were clearly demonstrated in mice (Alvarez et al., 2013). The dermis is also rich in micro-vascular systems that enable interaction between the cells of the immune system through network of regional lymph nodes.

Pigs vaccinated ID and IM had rising trends of GMTs at 14 dpv which increased gradually from 21 dpv to 28 dpv (p<0.01). It can be concluded that ID and IM vaccination induce comparable SN titers to CSF virus. SN titers of greater than 1:32 are acceptable protective levels for CSF (Suradhat and Damrongwatanapokin, 2003). In the present study maximum SN titers of 1:128 were seen as a result of ID and IM CSF vaccination. As compared with the 1ml IM vaccinated pigs, the pigs vaccinated ID with 0.2ml of the same vaccine had comparable antibody titers to IM group. We concluded that the ID route can be used as an alternative route for IM application of Lapinized Chinese-strain CSF vaccine. The cost of vaccine can be saved tremendously and can also increase a number of available of vaccine
doses, especially during CSF outbreaks where vaccine doses are demanding. However, it would be more convenient to use ID injecting tools rather than manually injecting the vaccine, since it is laborious and time consuming. The novel devices which guarantee less invasive, reproducible and easier injection will lead to a practical and widely use of intradermal vaccination strategy. A thorough investigation on using this technique in a larger number of pigs would give more confident result.

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