

UNIVERSITY OF THESSALY



SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY MEDICINE

CLINIC OF MEDICINE

**STUDY OF VACCINATION METHODS (INTRADERMAL, INTRAMUSCULAR) OF
SUCKLING PIGLETS AGAINST PRRSV-1 ON THEIR HEALTH AND
PERFORMANCE UNDER FIELD CONDITIONS**

A thesis submitted for the degree of Doctor of Philosophy

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CHAPTER I

Review of literature

1. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

Porcine Reproductive and Respiratory Syndrome (PRRS) is caused by an RNA virus referred to as PRRS virus (PRRSV), which is initially distinguished as genotypes PRRSV-1 (European) and PRRSV-2 (North American) (Zimmermann et al. 2019). According to the recent classification of the International Committee on Taxonomy of Viruses (ICTV 2021), previous genotypes are now considered to constitute two distinct species named *Betaarterivirus suid 1* and *Betaarterivirus suid 2* and classified within two different subgenera, *Eurpobartevirus* and *Ampobartevirus*, in the genus *Betaarterivirus* (subfamily *Variarterivirinae*, family *Arteriviridae*, suborder *Arnidovirinae*, order *Nidovirales*) (OIE 2021).

PRRS was first reported in North America in 1987 (Keffaber 1989) and was subsequently identified in both Europe and Asia in the 1990s (Wensvoort et al. 1991; Baron et al. 1992; Chang et al. 1993; Kuwahara et al. 1994). 1991 PRRSV-1 (European prototypic strain, Lelystad) was first isolated in the Netherlands (Wensvoort et al. 1991). PRRSV-2 (North American prototypic strain, VR2332) was isolated in the United States (Collins et al. 1992; McCullough et al. 1992; Wensvoort et al. 1992). The first report of PRRSV-1 in Greece was in 1993 (Xilouri 1993; Kyriakis et al. 1996), while recently PRSSV-2 strain was reported for the first time (Papatsiros et al. 2020). In Greece, PRRS appears enzootic, characterized by outbreaks throughout the year (Papatsiros et al. 2007).

Pigs can be infected through direct contact or indirectly via fomites. PRRSV exposure can happen through respiratory and oral routes, as well as through mucosae or percutaneously. Aerial transmission (short or long distance), coitus or insemination, ingestion, contact, and inoculation (often iatrogenically) are the methods involved. Vertical transmission is significant in the final trimester of gestation (Pileri and Mateu 2016). The development of viraemia and the body distribution of susceptible macrophages leads to the shedding of PRRSV by multiple routes. The presence of the virus in nasal secretions, saliva, urine, faeces, mammary gland

secretions, and semen is well documented in several studies (Christianson et al. 1993; Yoon et al. 1993; Rossow et al. 1994; Swenson et al. 1994; Rossow et al. 1995; Christopher-Hennings et al. 1995a; 1995b; Nielsen et al. 1997; Wills et al. 1997; Christopher-Hennings et al. 2001; Wagstrom et al. 2001; Prickett et al. 2008; Kang et al. 2010; Karniychuk et al. 2010; Kittawornrat et al. 2010).

After exposure to the virus, it initially replicates in macrophages in lymphoid tissues at the entry point. Then, it quickly spreads throughout the body via the lympho-haematic route. Following the viraemic phase, the virus is confined to secondary lymphoid tissues with reduced replication. Non-viraemic pigs were able to transmit the virus to naïve animals for up to 62 days after infection. Similarly, non-viraemic sows can transmit the infection to naïve sows between 49- and 86 days post-infection. Transmission of PRRSV from congenitally infected piglets to sentinel animals was observed up to 112 days after birth. Contagiousness is assumed to decrease over time, but under natural conditions, transmission can occur for up to 3 months in horizontally infected pigs. For congenitally infected animals, the contagious period may exceed this timeframe (Bierk et al. 2001; Wills et al. 2002; Rowland et al. 2003; Pileri and Mateu 2016).

When a previously unexposed herd is infected with PRRSV, all pigs are impacted, leading to a clinical outbreak. In a traditional farm, that covers all stages of production, the viral circulation initiates in one or more stages, often within the breeding herd. Subsequently, the virus spreads to all production stages within approximately 2-3 weeks. Sows can transmit the virus to their piglets either through the trans-placental route or via direct contact while nursing (Pileri and Mateu 2016). Piglets infected congenitally or very early in life can harbour the virus for several months and can contribute to the spread of the infection in the following production stages. As the infection progresses, the proportion of immune animals increases, and that of susceptible ones decreases. This leads to the decline phase of the epidemic in 1–5 months, depending on the herd size and the time needed to achieve protective immunity in most pigs

(Stevenson et al. 1993; Done et al. 1996; Nodelijk 2002; 2003; Evans et al. 2010). At this point, the infection usually becomes endemic, although, in some small farms, it may fade out just because of the exhaustion of susceptible pigs (Nodelijk et al. 2003). The transmission, infectious period, and susceptible pig population primarily dictate the virus's spontaneous extinction in a population. The likelihood of PRRSV fade-out decreases if the infection spreads to the farrowing house and piglets, as retrograde transmission from infected nurseries or finishers to the breeding herd becomes possible (Evans et al. 2010). On a farm, the maintenance of the infection is primarily sustained by a mix of animals with prolonged infection and the constant presence of susceptible pigs. The susceptibility of the pig population can arise from various factors, including the addition of new pigs, the birth of piglets from seronegative sows, the loss of passive immunity in young pigs, or the loss of active immunity in pigs previously infected (Nodelijk et al. 2003). PRRSV can persist on farms for extended periods, with the virus being transmitted among sows as well as through the movement of infected pigs from nurseries or finishing units (Evans et al., 2010). In chronically infected herds, it is commonly observed that most infections are subclinical (Pileri and Mateu 2016). In populations where PRRSV is consistently present, it can be transmitted within the breeding herd. This can lead to recurring episodes of reproductive diseases related to PRRSV and can also result in the infection of weaned piglet populations. This transmission can occur either vertically, from the sow to the piglet before weaning, or horizontally through direct contact between animals (Dee and Joo 1994). Evidence of PRRSV transmission in endemically infected breeding herds has been reported (Dee et al. 1996; Dee and Philips 1999). Within such populations, PRRSV-infected and naïve subpopulations of sows coexist, infected animals appear to cluster in small groups or exist as singletons, and naïve sows can produce PRRSV antibodies following exposure to the virus (Dee et al. 1996; 2000).

The clinical forms of PRRS include reproductive failures in breeding stock (delayed estrus returns, abortions, mummified fetuses, and weak-born piglets) and respiratory disease in nursery, weaning, and growing-finishing pigs characterized by reduced growth performance and increased mortality rate, mostly in neonatal pigs (Benfield et al. 1999; Feng et al. 2001; Rowland 2010; Papatsiros 2012; Rowland et al. 2012; Pileri and Mateu 2016).

Common symptoms include loss of appetite, fever, difficulty breathing, rapid breathing (polypnea), coughing, and decreased growth rates. In some cases, affected pigs may exhibit a bluish discolouration of the skin in their ears, abdomen, or vulva, which is known as "blue-eared disease." Acute cases can lead to death (Benfield et al. 1999; Papatsiros 2012; Zimmermann et al. 2019). Within affected herds, up to 50% of pigs may experience loss of appetite, up to 10% may have a fever, up to 5% can show cyanosis (bluish discolouration), and up to 30% may suffer from respiratory distress. Morbidity in weaning pigs can reach 30%, with a mortality rate of 5% to 10%. Nursery pigs often show symptoms such as respiratory distress and stunted growth, along with conjunctivitis, sneezing, and diarrhoea. The progression of the disease within a herd can span several days to a few weeks, with the overall course lasting 6 to 12 weeks (Benfield et al. 1999; Papatsiros 2012; Pileri and Mateu 2016; Zimmermann et al. 2019; Lunney et al. 2020).

Anorexia, lethargy, depression, and mild fever are common initial clinical symptoms observed in 5% to 50% of pregnant gilts and sows. This is often followed by an abrupt increase in early farrowing at 108 to 112 days gestation, late-term abortions, stillborn and mummified fetuses, partially autolyzed fetuses, weak neonates with high mortality rate within hours or days after birth, delayed return to estrus, and repeated breeding difficulties. Subsequently, middle gestation abortions and a significant rise in the number of mummified fetuses, early embryonic death, and infertility may be observed. In large herds, consecutive groups of 10% to 20% of gilts and sows may experience anorexia throughout 2 to 3 weeks. A small number of sows may

develop cyanosis of their ears, tails, vulvas, abdomens, and snouts, which is more common in European outbreaks and rare in North America. After the initial outbreak, severe reproductive failure can occur, including premature farrowing, late-term abortions, increased stillbirths, mummified fetuses, and weak neonates. This secondary phase of reproductive failure may last for 8 to 12 weeks, with stillbirth rates reaching 35% to 40%. Weak-born piglets typically perish within one week, contributing to a high preweaning mortality rate (Benfield et al. 1999; Lunney et al. 2010; Papatsiros 2012; Zimmermann et al. 2019).

2. PRRSV: impact on swine industry

Various economic analyses confirmed the vast financial impact of clinical outbreaks of PRRS on pig production (Pejsak et al. 1997; Holck and Polson 2003; Neumann et al. 2005; Papatsiros et al. 2007; Nieuwenhuis et al. 2012; Holtkamp et al. 2013).

PRRSV is a key pathogen involved in the porcine respiratory disease complex (PRDC) (Opriessnig et al. 2011; Chae et al. 2016; Zimmermann et al. 2019), causing also major economic losses worldwide. PRRSV can achieve synergism with other respiratory bacteria, such as *Mycoplasma hyopneumoniae* (*M. hyo*) and *Actinobacillus pleuropneumoniae* (App), even in PRRSV-vaccinated farms (Opriessnig et al. 2011; Fablet et al. 2012; Ruggeri et al. 2020). PRDC is associated with huge economic losses in the global pig industry (Holtkamp et al. 2013).

For example, studies have estimated that U.S. producers experience financial losses of \$664 million annually due to PRRSV (Neumann et al. 2005; Holtkamp et al. 2013). The situation in Europe is similar, with economic disease models determining the potential economic burden in various scenarios. The estimated annual losses range from €75,724 for farms slightly affected during nursing and fattening, to €650,090 for farms severely affected in all productive phases, such as those with 1,000 sows (Nathues et al. 2017). These costs align with the reported costs of the initial outbreak in The Netherlands, which were 98 euros per sow

(Nieuwenhuis et al. 2012). The outbreak resulted in a reduced number of sold pigs per sow, leading to economic losses ranging from €59 to €379 for one sow per 18-week period outbreak, with a mean loss of €126 per sow (Nieuwenhuis et al. 2012). Furthermore, a recent study in Germany found that the median farm budget for all farms was -31 euros per sow per year. However, if these farms had been PRRSV negative, the median simulated farm budget would have been 248 euros (Renken et al. 2021). The median total loss attributed to PRRS was 74,181 euros per farm per year, corresponding to a median total loss of 255 euros per sow per year. The impact of PRRS on farm profits was an average decrease of 19.1% and a worst-case decrease of 41% (Renken et al. 2021).

3. PRRSV and apoptosis

Pulmonary alveolar and/or intravascular macrophages are known as the major target cells for PRRSV in the lungs (Thanawongnuwech et al. 2000). There remains conflicting evidence regarding the impact of PRRSV infection on innate cell activation, antigen presentation, and the triggering of acquired immunity in host alveolar macrophages (Van Reeth et al. 1999; Labarque et al. 2000a; 2000b; 2000c; 2003; Van Gucht et al. 2003; Labarque et al. 2004). PRRSV infection triggers apoptosis in host cells, both *in vitro* and *in vivo*. This apoptotic process plays a crucial role in the development of PRRSV pathogenesis. Following exposure to the virus through the respiratory tract or oral ingestion, PRRSV primarily replicates in porcine macrophages. Specifically, pulmonary alveolar macrophages exhibit high levels of viral replication (Snijder et al. 2013; Fan 2019). As a result of virus replication, pulmonary alveolar macrophage function is impaired and the infiltration of inflammatory cells occurs, leading to interstitial pneumonia, which is the most common lesion type in PRRS infection (Vlahos et al. 2014; Ming et al. 2017; Wang et al. 2017).

It has been widely demonstrated that PRRSV causes apoptosis in different organs (lungs, testes, lymph nodes, and thymus) as well as in cell lines (Sur et al. 1997; Sirinarumitr

et al. 1998; Labarque et al. 1998; Sur et al. 1998; Labarque et al. 2001; Choi and Chae 2002; Feng et al. 2002; Labarque et al. 2003; Costers et al. 2008). Based on morphology, apoptotic cells in the lungs are believed to be alveolar and pulmonary intravascular macrophages and mononuclear cells in the alveolar septa. Double-labeling experiments have shown that most apoptotic cells are non-infected (Sirinarumitr et al. 1998; Sur et al. 1998). However, when the lungs are infected with PRRSV, there are unique cellular changes that can be attributed to apoptosis. These changes include a decrease in well-differentiated macrophages and an increase in new monocytes within the bronchoalveolar monocytes/macrophages population (Labarque et al. 2000). Apoptotic pathways can generally be categorized into two signaling cascades: the extrinsic pathway and the intrinsic pathway (Duprez et al. 2009).

Extrinsic, or “death” receptor-mediated apoptosis is initiated when a soluble ligand (such as tumor necrosis factor- TNF; Fas ligand, FasL; or TNF-related apoptosis-inducing ligand, TRAIL) binds to specific receptors on the cell surface (Nagata 1997; Ashkenazi and Dixit 1998). The cell-surface Fas receptor (Fas), also termed Apo-1 or CD95, is a member of the tumor necrosis factor (TNF) and nerve growth factor (NGF) family of receptors (Nagata and Goldstein 1995; Nagata 1997; Schulzeosthoff et al. 1998). Fas expression is observed in a variety of immune and non-immune cells (Watanabe-Fukunaga et al. 1992). Previous studies have covered the mechanisms underlying cell death induced by Fas/Fas ligand (FasL) interaction (Ashkenazi and Dixit 1998; Krammer 1998; Osborne 1998; Magnusson et al. 1999).

A soluble glycosylated protein (molecular mass, 50–60 kDa), angiotensinogen is the only precursor of angiotensin (Ang) peptides as well as the sole known substrate for renin (Chappell 2016). Angiotensinogen levels in blood circulation are relatively abundant (10–5 g or 10 µg/mL; 200 nM), especially compared with Ang II or Ang-(1–7), and for this reason, a minimal amount (<0.1 mL) of serum or plasma is needed to measure angiotensinogen (Chappell 2016). An ELISA method for detecting and quantifying angiotensinogen has been developed

and commercialized by Kobori and colleagues (2008). This method utilizes antibodies targeted at two different antigenic regions to capture and detect angiotensinogen. Ang II, an important component of the renin-angiotensin system (RAS) responsible for maintaining blood pressure and cardiovascular balance, is considered a systemic hormone produced through an enzymatic cascade (Lavoie et al. 2003). Renin is initially released from the juxtaglomerular cells of the kidneys into the bloodstream, where it enzymatically converts angiotensinogen into the decapeptide Ang I. Ang I can then undergo cleavage by angiotensin-converting enzyme (ACE) to produce the octapeptide Ang II in the pulmonary circulation (Campbell 1987; Johnston 1992; Wen 2012). Besides the well-known circulating RAS, Ang II is actively synthesized in various tissues and organs such as the heart, vasculature, kidney, and brain. This production occurs when angiotensinogen, renin, and ACE are present, highlighting the paracrine and intracrine effects of Ang II (Campbell 1987; Johnston 1992; Phillips et al. 1993; Vinson 1995; Wen 2012).

Ang II performs a crucial part in the inflammatory response, cell proliferation, migration, and apoptosis (Marchesi et al. 2008; Richardson et al. 2008; Hagiwara et al. 2009; Hernández et al. 2014). Previous studies have indicated that Ang II promotes apoptosis in both human and rat alveolar epithelial cells while inhibiting apoptosis in pulmonary endothelial cells during acute lung injury (Wang et al. 1999; Ji et al. 2015). The detrimental impact of PRRSV on the immune system of infected pigs is well-documented, as it induces apoptosis, necrosis, and necrosis-like apoptosis in lung and lymphoid organs (Labarque et al. 2003; Costers et al. 2008; Gómez-Laguna et al. 2013; Rodríguez-Gómez et al. 2014; Morgan et al. 2016).

In addition, Ang II is a second important factor of the Fas ligand (FasL) system in the lungs (Wang et al. 1999), while Fas/FasL is involved in PRRSV-induced apoptosis (Chang et al. 2007; Lee 2022). However, apoptosis could be induced in uninfected bystander cells by increased surface expression of FasL on surrounding cells caused by PRRSV infection, and as a result, these cells become sensitive to Fas-mediated apoptosis (Lee 2022).

There are scarce published findings on the role of Ang II after IM or ID vaccination of swine against PRRSV. The ACE promotes the conversion of Ang I into vasoconstrictor Ang II and can be found in many tissues including vascular tissue (Dezsö et al. 1989). ACE can promote vascular smooth muscle growth, capillary density, and consumption of oxygen, affecting both sympathetic and neuromuscular transmission and having a hypertrophic effect on skeletal muscle, improving contractile function (Jones et al. 2003; Dietze et al. 2008; Alves, et al. 2013; Vaughan et al. 2013; 2016).

4. PRRSV and Immunity

PRRSV is a virus that targets macrophages and can evade both innate and adaptive immune responses (Lunney et al. 2016). Once pigs are exposed to PRRSV, the virus replicates in alveolar macrophages and quickly spreads throughout the body through the lymphohematic route. This rapid spread results in an acute infection characterized by viremia that typically lasts for about a month (Pileri and Mateu 2016). Some studies have also reported a persistent infection of secondary lymphoid tissues that can last for approximately 150 days or longer (Wills et al. 2003). Viremia generally peaks around 7-10 days after infection and is mostly cleared by 28 days post-infection, although this can vary depending on the viral strain and the age of the pigs (van der Linden et al. 2003; Diaz et al. 2012). PRRSV is known to have immunosuppressive properties, which makes pigs more susceptible to secondary microbial infections (Done and Paton 1995). While many studies have investigated the interactions between PRRSV and host immune responses, most of them have focused on systemic immune responses using peripheral blood mononuclear cells (PBMC) and/or serum (Manickam et al. 2013). Previous research has shown that PRRSV-infected pigs often exhibit interstitial pneumonia as a major lung lesion, with significantly reduced numbers of alveolar macrophages in bronchoalveolar lavage (BAL) and lung tissue samples (Bordet et al. 2018).

The immune system of pigs can effectively clear the PRRSV virus during infection (Wesley et al. 2006). To achieve this, the innate immune system of pigs needs to be properly stimulated to promote the development of protective adaptive immunity against PRRSV. Alveolar macrophages in the lungs are the primary sites for PRRSV replication and are part of the respiratory DC/macrophage network. This network is responsible for detecting foreign antigens, controlling inflammation, and initiating adaptive immune responses (Maisonnasse et al. 2015). Certain subsets of DCs within this network have specialized functions and are resistant to PRRSV infection (Maisonnasse et al. 2016; Bordet et al. 2018). T cells also play a crucial role in clearing infected cells from the body and regulating immune responses specific to PRRSV antigens (Loving et al. 2015).

The ability of PRRSV to escape innate immune responses in its replicating cells is well known. It primarily affects macrophages and monocyte-derived cells. This escape mechanism involves inhibiting type I interferon and other innate immune responses (Lunney et al. 2016; Ma et al. 2021). Nevertheless, plasmacytoid dendritic cells can sense PRRSV and PRRSV-infected cells and contribute to innate sensing of the virus (Baumann et al. 2013; Garcia-Nicolas et al. 2016). At the level of adaptive immune responses to PRRSV, pigs can mount a protective immune response, mainly against similar virus strains; but this virus is described to induce both delayed and weak neutralizing antibody and T-cell responses (Lunney et al. 2016; Rahe and Murtaugh 2017). Several factors are believed to contribute to the slow development of adaptive immune responses against PRRSV. These factors include the heavy glycosylation of viral surface proteins, which may shield epitopes and hinder recognition by immune cells. The involvement of regulatory T cells is also considered a potential factor. Additionally, PRRSV has been observed to target the thymus and interact with the monocyte-macrophage system. Despite these findings, the exact reasons why PRRSV is challenging for the immune system to

control remain unclear (Loving et al. 2015; Lunney et al. 2016; Stoina et al. 2019; Wang et al. 2020).

5. PRRSV - vaccines

Regarding PRRS vaccination, several commercial vaccines are available on the market based on either a modified live virus (MLV) or a killed virus (KV). The control of prevention strategies for a PRRSV infection includes mainly the vaccinations of sows and/or piglets with modified live virus (MLV) vaccines. In sows, killed vaccines (KV) are also used (Papatsiros et al. 2006; Papatsiros 2010; Murtaugh et al. 2011; Papatsiros 2012; Renukaradhya et al. 2015). Nowadays, MLV vaccines are widely used in practice because they elicit a stronger and more complete immune response compared to KV vaccines (Labarque et al. 2003; Papatsiros et al. 2006; Papatsiros 2012; Nan et al. 2017; Chae et al. 2021).

Five commercial MLV vaccines based on PRRSV-1 are available in Europe: a) Porcilis® PRRS for sows and piglets (MSD Animal Health), b) UNISTRAIN® PRRS for sows and piglets (Hipra), c) ReproCyc® PRRS EU for sow (Boehringer Ingelheim), d) Ingelvac PRRS FLEX® EU for piglets (Boehringer Ingelheim) and Suvaxyn PRRS MLV for sows and piglets (Zoetis). Although MLV vaccines offer good virological and clinical protection against homologous challenges, they only provide partial immunity against heterologous challenges. However, these vaccines are still effective in reducing abortions and mortality in newborn piglets, as well as improving reproductive parameters in PRRSV-infected sows (Benfield et al. 1999; Nilubol et al. 2004; Scotti et al. 2006). Vaccinating pigs with an MLV vaccine has been shown to lower viremia levels and reduce lung lesion severity in response to experimental infection with a heterologous Lelystad-like (subtype 1) strain (Van Woensel et al. 1998; Diaz et al. 2005; Martelli et al. 2009; Kim et al. 2015). In addition, an MLV vaccine provided partial protection against the Italian highly pathogenic PRRSV-1 subtype 1 strain based on clinical, virological, and pathological analysis (Canellia et al. 2018).

Four MLV commercial vaccines in Europe tested provided virtually the same clinical partial protection against infection from the highly pathogenic PRRSV-1 subtype 3 Lena strain by reducing the fever period duration in all studies (Trus et al. 2014; Bonckaert et al. 2016; Renson et al. 2017). While evaluating virological parameters, a significant reduction in viremia with two MLV vaccines was shown (Trus et al. 2014; Renson et al. 2017), while a significant decrease in viremia was not observed in the remaining two MLV vaccines (Bonckaert et al. 2016; Renson et al. 2017).

PRRSV, a major cause of porcine respiratory disease complex (PRDC), can worsen respiratory issues when combined with other bacteria or viruses in vaccinated growing/finishing pigs (Opriessnig et al. 2011). Thus, vaccination against PRRSV is important for reducing decreased performance and feed efficiency in this group (Opriessnig et al. 2011; Linhares et al. 2016). Nonetheless, there is a lack of research on the effectiveness of PRRSV vaccination during suckling age in real-world conditions and its impact on the performance of growing/finishing pigs (Park et al. 2014; Moura et al. 2019; Díaz et al. 2020). However, previous studies have shown that PRRSV vaccination can lead to improved growth performance in practical settings (Lyoo et al. 2016; Thomann et al. 2020).

Nevertheless, PRRSV vaccine efficacy studies conducted for the growth performance of growing/finishing pigs are rarely reported because nursery pigs are more susceptible than adult pigs to develop the disease and management conditions, such as biosecurity and various pathogen factors, are more complex in a growing/finishing herd (Park et al. 2014).

6. PRRSV and Intradermal (ID) vaccination

The development of needle-free injection systems dates back to the 1930s (Hingson et al. 1952). These systems have been utilized in human medicine for the delivery of insulin, anaesthetics, growth hormones, and vaccines (Katoulis et al. 1989; Verhagen et al. 1995; Jimenez et al. 2006; Weniger and Glenn 2013). The skin, with its abundance of

immunocompetent cells like Langerhans cells and dermal dendritic cells that specialize in antigen uptake and presentation, is an appealing vaccination target due to its strong immune response capabilities (Bangert et al. 2011).

In veterinary medicine, intradermal vaccination has gained significant interest in the past decade. This needle-free method of administering antigens is less invasive and less painful compared to traditional subcutaneous or intramuscular injections with a needle. By utilizing intradermal needle-free devices, intradermal (ID) vaccination offers several important benefits. Firstly, it greatly improves the welfare of the animals being vaccinated. The needle-free administration is less painful and minimizes the likelihood of developing histological lesions at the injection site. This leads to a more comfortable experience for the animals. Secondly, ID vaccination helps prevent the iatrogenic transmission of pathogens in vaccinated pigs. With no needle involved, there is a reduced risk of contaminating the animal with pathogens during the vaccination process. Moreover, by eliminating the use of needles, accidental worker self-injections are also avoided. This further enhances safety for the individuals administering the vaccines. Finally, ID vaccination contributes to improving the quality of the carcass. Since there are no needle-induced injection site lesions or broken needles, the resulting pork carcasses remain intact and free from any residual needle fragments. Overall, the adoption of intradermal vaccination using intradermal needle-free devices offers numerous advantages such as enhanced animal welfare, prevention of pathogen transmission, increased safety for workers, and improved carcass quality (Hafer et al. 1996; Sarno 2000; Kenney 2004; Stout et al. 2004; Mitragotri 2005; 2006; Saia et al. 2010; Baker et al. 2012).

Intradermal (ID) vaccination has gained increasing interest in swine medicine as an alternative to intramuscular (IM) vaccination due to the advantage of aiming at antigen-presenting cells in the epidermis close to the skin-draining lymph nodes (Romani et al. 2012; Teunissen et al. 2012). This administration induces a more rapid and direct response of the

vaccinated animal to the antigen induced by the vaccine. Previous safety and efficacy studies with an ID vaccination have been reported against various respiratory pathogens, such as PRRSV (Martelli et al. 2007; 2009; Baker et al. 2012; Stadler et al. 2018; Madapong et al. 2021), porcine circovirus type 2 (PCV2) (Sno et al. 2016), Aujeszky's disease (Visser et al. 1994; Ferrari et al. 2011), and *M. hyo* (Tassis et al. 2012; Beffort et al. 2017).

Previous studies showed that an ID vaccination against PRRSV has at least a similar immunological effect compared to IM (Martelli et al. 2007; 2009), as pigs intradermally (ID) vaccinated did not show any differences in terms of viral load in their blood and clinical signs when compared to animals IM vaccinated. Other studies also reported that an ID vaccination could provide a similar protective immunity compared with an IM vaccination, under experimental conditions (Jiang et al. 2021). Moreover, the ID administration of a commercial PRRSV-1 MLV vaccine can induce efficient protection in pigs later exposed to a genetically diverse PRRSV strain, under field conditions (Martelli et al. 2007). In the same study by Martelli et al. (2007) advantages such as safety for the executor, painlessness for the pig, absence of histological lesions, and no iatrogenic transmission of pathogens were reported. Moreover, a previous study suggested that a PRRSV-1 MLV vaccine administered by either IM or ID can provide partial heterologous protection after challenge with a highly pathogenic PRRSV-2 strain, either alone or combined with PRRSV-1, as demonstrated by less lung tissue lesions and reduced viremia (Madapong et al. 2020).

Even though the ID vaccination of piglets against PRRSV is a common practice, there is no literature investigating, under field conditions, the effect of an ID vaccination of suckling piglets against PRRSV on the growth performance parameters up to the finishing stage. Recent studies have highlighted the notable advantages of ID vaccination over IM vaccination for piglets' welfare. Research has shown that ID vaccination against *M. hyo* and PCV2 resulted in fewer post-vaccination behavior and activity changes in suckling piglets compared to

intramuscular vaccination (Göller et al. 2020; Temple et al. 2020). European consumers, who actively seek animal-friendly products, place a high value on higher welfare standards at the farm level (Pejman et al. 2019). As a result, alternative routes to the traditional intramuscular vaccination, such as the PRRS, have been developed. Specifically, IM vaccination against the PRRS has been confirmed as an effective alternative, as it elicits similar humoral and cell-mediated immune responses as the intramuscular route (Madapong et al. 2020). Additionally, ID vaccination against the PRRSV has demonstrated a better response in certain parameters related to pregnant sows' behavior and stress indicators (Temple et al. 2017).

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CHAPTER II

Evaluation of Intradermal PRRSV MLV Vaccination of Suckling Piglets on Health and Performance Parameters under Field Conditions

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) induces mainly respiratory disease in weaning and growing pigs, as well as reproductive failures in the breeding stock [1,2,3]. Studies have shown the important financial impact which outbreaks of PRRS have on the global swine industry [1,4,5,6]. PRRS virus (PRRSV) is a key pathogen involved in the porcine respiratory disease complex (PRDC) [7,8], causing also major economic losses worldwide. PRRSV can achieve synergism with other respiratory bacteria, such as *Mycoplasma hyopneumoniae* (*M. hyo*) and *Actinobacillus pleuropneumoniae* (App), even in PRRSV-vaccinated farms [7,9,10].

The control of prevention strategies for a PRRSV infection includes mainly the vaccinations of sows and/or piglets with modified live virus (MLV) vaccines. In sows, killed vaccines (KV) are also used [1,11,12,13,14,15]. The control of PRRS by vaccination is important also to decrease economic losses due to a decreased performance and feed efficiency [7,16]. Studies on the effectiveness of the PRRSV vaccination at the suckling age under field conditions, including the investigation of its impact on the performance parameters of growing/finishing pigs, are rarely reported [17,18,19]. However, previous studies reported that a PRRSV vaccination is associated with an improvement in the growth performance, under field conditions [20,21].

Intradermal (ID) vaccination has gained increasing interest in swine medicine as an alternative to intramuscular (IM) vaccination due to the advantage of aiming at antigen-presenting cells in the epidermis close to the skin-draining lymph nodes [22,23]. Many safety and efficacy studies with an ID vaccination have been reported against various respiratory pathogens, such as PRRSV [24,25,26,27,28], porcine circovirus type 2 (PCV2) [29], Aujeszky's disease [30], and *M. hyo* [31].

Previous studies showed that an ID vaccination against PRRSV has at least a similar immunological effect compared to IM [24,25,27]. Other studies also reported that an ID vaccination could provide a similar protective immunity compared with an IM vaccination, under experimental conditions [32]. Moreover, the ID administration of a commercial PRRSV MLV vaccine can induce efficient protection in pigs later exposed to a genetically diverse PRRSV strain, under field conditions [24]. Finally, an ID vaccination that is applied with intradermal needle-free devices has important benefits in terms of the animal's welfare as a needle-free administration (e.g., a less invasive and less painful administration and the absence of histological lesions on the point of application), the prevention of the iatrogenic transmission of pathogens in vaccinated pigs, the elimination of accidental worker self-injections, and the carcass' quality (e.g., a lack of needle-induced injection site lesions or broken needles and residual needle fragments in pork carcasses) [26,33,34,35,36,37]. Even though the ID vaccination of piglets against PRRSV is a common practice, there is no literature investigating, under field conditions, the effect of an ID vaccination of suckling piglets against PRRSV on the growth performance parameters up to the finishing stage.

This field study aimed to evaluate the impact of the ID vaccination of piglets with a commercial PRRSV MLV vaccine in comparison to an IM vaccination on their health status and performance until they reached their slaughter age, under field conditions, in a farm suffering from an active PRRSV infection.

2. Materials and Methods

2.1. Animal Ethics and Experimental Farm

All procedures regarding animal care, handling, treatment, and use were approved by the Ethical Committee of the Faculty of Veterinary Medicine, School of Health Sciences, University of Thessaly (permission number: 98/19-12-2019). The owner of the farm the

research took place on was thoroughly informed and provided written consent for the use of the farm animals in the described experimentation.

The current trial was carried out in a farrow-to-finish commercial pig farm located in Central Greece. The productive capacity of the farm was 150 commercial hybrid sows (Large White x Landrace, Topigs Norsvin). A grandparent nucleus was maintained on the farm for producing breeding gilts. The herd practiced a 1-week batch production system. At weaning (28 ± 3 days), the grouping of the piglets at flat-deck nurseries was equally based on their body weight and sex, applying a stratified randomization procedure. The semen used for the artificial insemination was collected from commercial hybrid boars (Topigs Norsvin) that were maintained on the farm.

The vaccination protocol of farm sows included routine vaccinations against PRRSV, Aujeszky's disease virus, swine parvovirus, atrophic rhinitis, swine erysipelas, *Escherichia coli*, and *Clostridium perfringens*. Piglets were vaccinated against PCV2 and *M. hyo* at the age of 3 weeks. The routine antiparasitic program of sows included a single IM administration of ivermectin two weeks before farrowing. As for PRRSV, the sows were vaccinated following the 6th day of lactation and the 60th day of the gestation vaccination plan, the gilts were vaccinated twice with a three-week gap between the doses before entering productive life, and the boars were vaccinated biannually as a farm routine. For the immunization of the breeding stock, the Porcilis PRRS vaccine by MSD Animal Health was used, the same commercial product which was utilized in our trial. At the onset of the current experimentation, no piglets on this farm were ever vaccinated against PRRSV before.

The feed provided was homemade and based on corn/barley/wheat-soya, using an automatic feeding system. Animals participating in the trial had at-will access to drinking water and an automatic temperature and humidity system-controlled housing facility.

2.2. Farm History-Pre-Trial Period

Four weeks prior to the onset of the experimentation, blood sampling was performed on different ages; sows and gilts (12 total blood samples from gilts and sows in gestation and lactation stages), suckling and weaned piglets, and growing and finishing pigs (8 blood samples at the ages of 2, 4, 7, 10, 13, 17, and 21 weeks). Testing via qRT-PCR revealed the farm's positivity for PRRSV during the pre-trial period, with the following prevalence: 100% at 4, 7, 10, and 13 weeks, 58% at 17 weeks, and 22% at 21 weeks. Based on the complete ORF 5 sequencing and bioinformatics analysis, it was found that the PRRSV strain detected in the farm had a 90.7% sequence identity with the DV vaccine strain. The PRRSV genome was not detected at the age of 2 weeks. In addition, the examination of the blood samples from unvaccinated suckling piglets (7, 14, 21, and 28 days of age) for PRRSV-specific antibodies via ELISA showed low levels of maternal antibodies at 14 days of age and low levels or the absence of maternal antibodies at 21 and 28 days of age.

The main clinical problems at the weaning and fattening stage were the increased morbidity associated with respiratory symptoms and an increased mortality rate, due mainly to secondary co-infections. Piglets belonging to batches born prior to the beginning of the field trial were vaccinated only against PCV2 and *M. hyo* at the age of 3 weeks.

2.3. Experimental Animals

In total, 187 healthy suckling piglets (2 weeks of age) were included in the study and were assigned to 4 groups. Four replicates were formed in each group, in each of which 11–12 piglets were allocated), as shown in **Table 1**. The diseased piglets with a significantly low body weight (BW) or congenital flaws (e.g., hernia) were excluded from the trial.

Table 1. Vaccinated and non-vaccinated groups during the trial (replicates and numbers of the piglets).

Replicates	Groups			
	Group A PRRSV ID (0.2 mL Porcilis PRRS-ID)	Group B PRRSV IM (2 mL Porcilis PRRS-IM)	Group C Control ID (0.2 mL of Diluvac Forte-ID)	Group D Control IM (2 mL of Diluvac Forte-IM)
	Number of Piglets			
1	12	12	11	12
2	11	12	12	11
3	12	11	12	12
4	12	12	11	12
Total	47	47	46	47

The piglets were placed individually (within the litters) in the test groups equally based on the BW and sex, as well as the parity number of their sows (parities 1 to 5). At the time of admission, the piglets were ear-tagged. Four unique numbered ear tags were used for their identification and the numbers were recorded. The color of the tags varied by the production batch and the lost tags were replaced. The pigs of the test and control groups were mixed in different pens in the same nursery room. Then, all of the pigs were housed as usual in shared pens in all production stages from nursery to finishing age.

2.4. Experimental Material, Dosage, and Administration

The vaccine utilized in the trial for the immunization of the piglets against PRRSV was Porcilis® PRRS (MSD Animal Health). It is a commercially available, live attenuated lyophilized vaccine, containing per dose of 2 mL (IM application) or 0.2 mL (ID application) of reconstituted vaccine, the active substance (lyophilisate), i.e., the live attenuated PRRSV-1 strain DV at titers $10^{4.0}$ – $10^{6.3}$ TCID₅₀ (50% tissue culture infective dose), along with the Diluvac Forte® adjuvant (solvent) that contains dl- α tocopheryl acetate at a concentration of 75 mg/mL.

The piglets included in the test groups (A and B) were vaccinated at the age of 2 weeks with 1 dose of Porcilis® PRRS, diluted in 0.2 mL (group A, ID) or 2 mL (group B, IM) of Diluvac Forte®. The piglets included in the control groups (C and D) were not vaccinated against PRRSV and only Diluvac Forte® was administered (0.2 mL for group C, or 2 mL for group D) (Table 1). An IDAL (IntraDermal Application of Liquids) vaccinating device was used for the needle-free intradermal injection of piglets belonging to ID groups (A and C). For the administration of the piglets belonging to the IM groups (B and D), an automatic syringe, with a fixed 2 mL volume, was used. A new, clean 18 G needle (size approx. 0.9 × 13 mm) was used for each IM trial group.

No other vaccinations or vaccines against PRRSV or other veterinary products (concurrently or simultaneously) were applied within 14 days before or 7 days after the administration of Porcilis® PRRS. The piglets were vaccinated against PCV2 and *M. hyo* with a single dose vaccine at the age of 3 weeks. All the treatments of the growing–fattening pigs (preventive oral or injectable treatments and vaccinations) were recorded for the treatment and control groups.

2.5. Samplings and Laboratory Examinations

Blood samples were collected from the same 3 identified pigs belonging to each group for each of the 4 replicates. Sampling was performed at the ages of 4, 7, 10, 13, 17, and 21 weeks. Serological testing for PRRS was performed on each serum sample according to the manufacturer's recommendations (IDEXX PRRS X3 Ab Test). Sample/Positive (S/P) ratios were used as the estimates of the antibody titers. Serum blood samples were also subjected to a nucleic acid extraction using the PureLink® Viral RNA/DNA Mini Kit (Invitrogen). The extracts were tested for the PRRSV genome by qRT-PCR using a previously described TaqMan probe-based qRT-PCR method [38]. Ct (cycle threshold) values were used for the estimation of viremia titers.

2.6. Records

2.6.1. Local and Systemic Reactions

The investigators consistently checked all the study animals individually for reactions and side effects post-vaccination, on day 0 and at 1, 4, 7, and 14 days post-vaccination. That was a group observation that did not require the individual inspection and palpation of each piglet (which was not practically possible with a large number of animals in the study).

2.6.2. Performance Parameters

To obtain the average daily gain (ADG) (g/pig/day), each different group per replicate was weighed on admission day, on weaning day, at the end of the nursery period, and at their slaughter age. The study animals were weighted in study groups and the calculation of the means as an individual measurement was not possible due to the farm routine. In addition, the age of slaughter was recorded.

2.6.3. Mortality

At any time after an admission, a post-mortem examination was presented on a piglet that died or was culled. The percentage of mortality was recorded at different periods (nursing, growing, and finishing stages).

2.6.4. Lung Lesion Score (LLS) and Pleurisy Lesion Score (PLS)

All the lungs of the pigs included in the trial were examined and scored for their typical gross lesions according to the method of Goodwin and Whittlestone (1993). The lesions of cranio-ventral pulmonary consolidation (LLS ranged from 0 to 55 at maximum) of each tested lung were the addition of the proportion of each lobe surface with signs of typical inflammation, multiplied by the weighting of each lobe. Moreover, the pleurisy lesions (PLS) were evaluated as follows: 0—no pleurisy lesions, 1—topical adhesions (spots), and 2—larger adhesions. To

prevent bias, one pathologist responsible for the lung examination at the slaughterhouse was blinded for the treatment group of the animals.

2.7. Statistical Analysis and Study Power Analysis

The data were presented as the mean $\pm$ standard deviation. A statistical analysis was done using IBM SPSS 21 (IBM Corp., Armonk, NY, USA). A data analysis among the groups was performed using the Kruskal–Wallis H test, and the Mann–Whitney U test was used for multiple comparisons. The comparisons within the groups were performed using the Friedman test and the Wilcoxon Signed Rank test. Significant differences were considered below the 0.05 level. A power analysis was done with G*Power software (version 3.1) [39]. The total sample size and power analysis were estimated for each one of the measured study variables (the ELISA, qRT-PCR results, body weight, etc.).

3. Results

3.1. Local and Systemic Reactions

No significant adverse reactions were noticed in vaccinated piglets on day 0 and at 1, 4, 7, and 14 days post-vaccination.

3.2. Laboratory Examination

3.2.1. ELISA Results

The results of the ELISA testing in the serum samples are shown in Table 2 and Figure 1. After 13 weeks, the seropositivity of all animals in the four groups was observed, possibly due to a natural infection, but the level of specific PRRSV antibodies at the end of the finishing stage (21 weeks) was significantly lower in group A in comparison to the other groups ($p < 0.05$ compared to groups B and D; $p < 0.01$ compared to group C). Significant differences between the ID vaccinated group A and the control group D were also observed at 10 and 17 weeks ($p < 0.05$). The within-group comparisons also revealed significant differences through time for all

four groups (Table 2). These findings, along with the observed viral loads (Section 3.2.2), suggest that the vaccinated animals of group A had a more efficient protection, as higher antibody levels in the other groups probably indicate a more prolonged viremia and/or higher PRRSV load, which results in a more intense immune response.

Table 2. ELISA testing results in serum samples at 4, 7, 10, 13, 17, and 21 weeks of age.

Group	Age (Weeks)					
	No. of Positive/Total					
	Mean S/P Ratio $\pm$ SD of Positive Samples					
	4	7	10	13	17	21
Group A (PRRSV ID)	11/12	8/12	10/12	12/12	12/12	12/12
	1.22 $\pm$ 0.46 ^{b,x}	0.79 $\pm$ 0.24 ^{c,x}	1.38 $\pm$ 0.82 ^{b,y}	1.79 $\pm$ 1.12 ^{a,x}	1.87 $\pm$ 0.91 ^{a,y}	1.42 $\pm$ 0.72 ^{ab,z}
Group B (PRRSV IM)	12/12	5/11 *	11/11	11/11	11/11	11/11
	1.25 $\pm$ 0.95 ^{b,x}	0.66 $\pm$ 0.21 ^{c,x}	1.71 $\pm$ 0.92 ^{ab,xy}	2.11 $\pm$ 0.75 ^{a,x}	2.14 $\pm$ 0.61 ^{a,xy}	2.12 $\pm$ 0.61 ^{a,y}
Group C (Control ID)	9/12	3/12	12/12	12/12	11/11 *	11/11
	0.98 $\pm$ 0.48 ^{c,x}	1.63 $\pm$ 1.14 ^{c,x}	1.66 $\pm$ 0.78 ^{b,xy}	2.39 $\pm$ 0.98 ^{a,x}	2.48 $\pm$ 0.60 ^{a,xy}	2.86 $\pm$ 0.86 ^{a,x}
Group D (Control IM)	8/12	7/12	12/12	12/12	12/12	12/12
	1.54 $\pm$ 0.60 ^{b,x}	0.99 $\pm$ 0.70 ^{b,x}	2.05 $\pm$ 0.83 ^{a,x}	2.48 $\pm$ 0.84 ^{a,x}	2.70 $\pm$ 0.81 ^{a,x}	2.35 $\pm$ 1.18 ^{a,y}

* One dead animal. ^{a-c} Comparison of ELISA testing results within groups (i.e., within a row). Values with the same superscript are not significantly different from each other ($p \geq 0.05$), but they differ significantly ($p < 0.05$) from values with a different superscript. ^{x-z} Comparison of ELISA testing results between groups (i.e., within the column). Values with the same superscript are not significantly different from each other ($p \geq 0.05$), but they differ significantly ($p < 0.05$) from values with a different superscript.

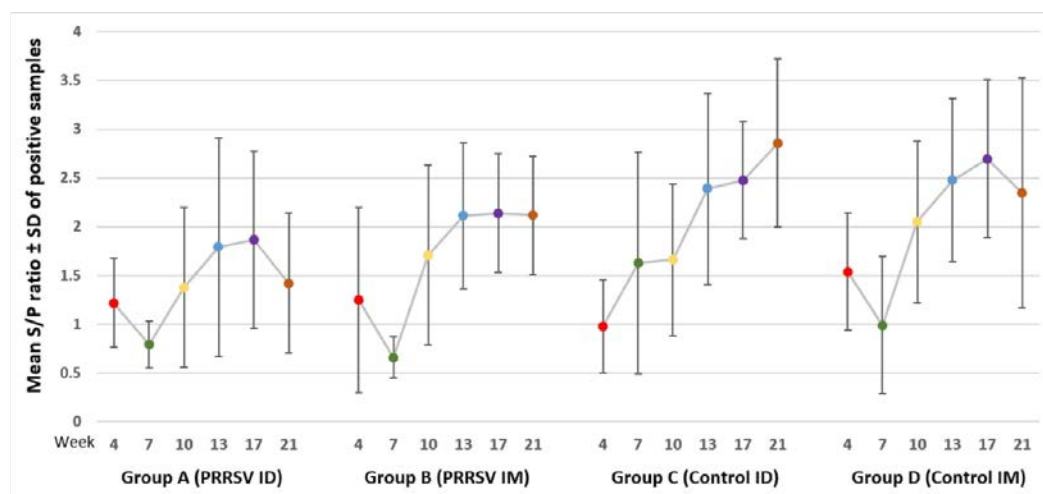


Figure 1. The results of ELISA examination (Mean S/P ratio $\pm$ SD) antibody-positive serum samples.

3.2.2. qRT-PCR Results

The results of the qRT-PCR examination of the blood samples are shown in Table 3 and Figure 2. Only 1/12 PRRSV-positive animal was found in each group at 4 weeks, and the animals of all the 4 groups were not viremic at 17 and 21 weeks. It was shown that the percentage of

PRRSV-positive samples at 10 weeks was lower in group A (75%) in comparison to the other groups (91–100%). A similar observation regarding the percentage of PRRSV-positive animals in the ID vaccinated group A compared to the other groups was also observed at 7 weeks (42% for group A vs. 55–83% for the other groups) and 13 weeks (33% for group A vs. 36–58% for the other groups). Moreover, based on the qRT-PCR Ct category [1 = weak positive (Ct $\geq$ 35), 2 = positive (Ct < 35, $\geq$ 25), 3 = strong positive (Ct < 25)], high viremia titers were noticed in non-vaccinated groups (group C and group D) at 7, 10, and 13 weeks. The statistical analysis of qRT-PCR testing results between the groups revealed significant viral load differences only at 10 weeks in group A vs. group C ($p < 0.01$) and group B vs. group C ($p < 0.05$). The within-group comparisons also revealed significant differences through time for all four groups (Table 3).

Table 3. Results of qRT-PCR in serum samples at ages of 4, 7, 10, 13, 17, and 21 weeks.

Group	Age (Weeks)				
	No. of Positive/Total				
	Mean Ct-Value $\pm$ SD of Positive Samples No. of Positive/qRT-PCR Ct Category **				
	4	7	10	13	17
Group A (PRRSV ID)	1/12	5/12	9/12	4/12	0/12
	39.16 $\pm$ 0.00 ^{a,x}	27.25 $\pm$ 2.65 ^{b,x}	30.14 $\pm$ 2.14 ^{b,x}	38.40 $\pm$ 1.27 ^{a,x}	
	Cat.1: 1	Cat.2: 4, Cat.3: 1	Cat.2: 9	Cat.1: 4	
Group B (PRRSV IM)	1/12	6/11 *	10/11	4/11	0/11
	35.18 $\pm$ 0.00 ^{a,x}	30.08 $\pm$ 5.39 ^{b,x}	31.62 $\pm$ 2.85 ^{c,x}	34.62 $\pm$ 2.90 ^{ab,x}	
	Cat.1: 1	Cat.1: 1, Cat. 2: 4, Cat.3: 1	Cat.1: 1, Cat.2: 9	Cat.1: 2, Cat.2: 2	
Group C (Control ID)	1/12	9/12	12/12	7/12	0/11 *
	35.31 $\pm$ 0.00 ^{a,x}	27.04 $\pm$ 3.48 ^{c,x}	28.00 $\pm$ 3.15 ^{c,y}	35.69 $\pm$ 1.81 ^{b,x}	
	Cat.1: 1	Cat.2: 6, Cat.3: 3	Cat.2: 8, Cat.3: 4	Cat.1: 4, Cat.2: 3	
Group D (Control IM)	1/12	10/12	11/12	7/12	0/12
	35.11 $\pm$ 0.00 ^{a,x}	27.37 $\pm$ 3.31 ^{c,x}	30.49 $\pm$ 3.55 ^{c,xy}	33.87 $\pm$ 2.21 ^{b,x}	
	Cat.1: 1	Cat.2: 8, Cat.3: 2	Cat.2: 9, Cat.3: 2	Cat.1:4, Cat.2: 3	

* One dead animal; ** qRT-PCR Ct value category (Cat.), 1 = weak positive (Ct $\geq$ 35), 2 = positive (Ct < 35, $\geq$ 25), 3 = strong positive (Ct < 25). ^{a-c} Comparison of qRT-PCR testing results within groups (i.e., within row). Values with the same superscript are not significantly different from each other ($p \geq 0.05$), but they differ significantly ($p < 0.05$) from values with a different superscript. ^{x,y} Comparison of qRT-PCR testing results between groups (i.e., within column). Values with the same superscript are not significantly different from each other ($p \geq 0.05$), but they differ significantly ($p < 0.05$) from values with a different superscript.

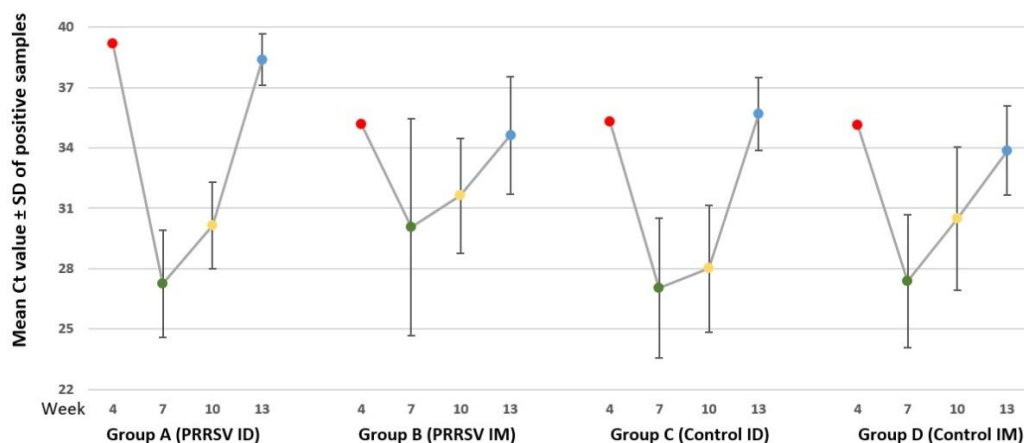


Figure 2. Ct values (mean $\pm$ SD) of qRT-PCR in serum samples at 4, 7, 10, and 13 weeks of age.

3.3. Performance Parameters

Table 4 summarizes the values for the performance parameters (the BW, age of slaughter, and ADG) per group at different periods (on admission, on weaning, at the end of the nursery period, and before their slaughter). In terms of the BW comparison at the same time between the groups, there was a statistically significantly higher BW only between the ID vaccinated group A and the non-vaccinated group and that observed before the slaughter ($p < 0.05$). The differences in the age of slaughter were not significant. The ADG was significantly higher in both vaccinated groups (A and B) in comparison to non-vaccinated groups (C and D) ($p < 0.05$).

Table 4. Performance parameters (BW, age of slaughter, ADG) (mean $\pm$ SD).

Groups	Time *	BW (kg)	Age of Slaughter (Days)	ADG (kg)
Group A	1	3.85 $\pm$ 0.22 ^a	154.50 $\pm$ 1.73 ^f	0.75 $\pm$ 0.01 ^g
	2	7.71 $\pm$ 0.38 ^b		
	3	27.03 $\pm$ 1.05 ^c		
	4	116.53 $\pm$ 1.16 ^d		
Group B	1	3.88 $\pm$ 0.19 ^a	156.50 $\pm$ 1.29 ^f	0.74 $\pm$ 0.01 ^g
	2	7.71 $\pm$ 0.42 ^b		
	3	27.05 $\pm$ 1.27 ^c		
	4	115.03 $\pm$ 0.43 ^{de}		
Group C	1	3.93 $\pm$ 0.17 ^a	158.75 $\pm$ 2.22 ^f	0.72 $\pm$ 0.01 ^h
	2	7.68 $\pm$ 0.44 ^b		
	3	26.38 $\pm$ 1.05 ^c		
	4	113.65 $\pm$ 0.60 ^e		
Group D	1	3.87 $\pm$ 0.03 ^a	160.25 $\pm$ 1.71 ^f	0.71 $\pm$ 0.01 ^h
	2	7.51 $\pm$ 0.23 ^b		
	3	26.23 $\pm$ 0.71 ^c		
	4	114.25 $\pm$ 0.96 ^{de}		

* 1: admission, 2: weaning, 3: end of nursery period, 4: slaughter. ^{a-e} Comparison of BW between groups (i.e., within the column) and at different times (i.e., 1: a, 2: b, 3: c, and 4: d-e). The same superscript indicates non-significant differences ($p \geq 0.05$) between groups, for the same time. Different superscripts indicate significant differences ($p < 0.05$) between groups, for the same time. ^{f-h} Comparison of age of slaughter (f) and ADG (g, h) between groups (i.e., within the column). Values with the same superscript are not significantly different from each other ($p \geq 0.05$), but they differ significantly ($p < 0.05$) from values with a different superscript.

3.4. Mortality

The mortality per group at different periods (nursery stage, growing stage, fattening stage) is presented in Table 5. No mortality was noticed in all of the groups between the vaccination time and the weaning day. The mortality was significantly lower in vaccinated groups (A and B) in comparison to non-vaccinated groups (C and D) at the finishing stage ($p < 0.05$). A similar finding regarding mortality though the whole period ($p < 0.01$) was observed. In addition, the percentage of mortality at the growing stage was significantly lower in the ID-vaccinated group A compared to that of the non-vaccinated group D ($p < 0.05$).

Table 5. The mortality per group at different periods.

Groups	Mortality % (No. of Dead/Total)			
	Nursery Stage	Growing Stage	Fattening Stage	Total Period
Group A	2.1 (1/47) ^a	0 (0/46) ^b	4.3 (2/46) ^b	6.3 (3/47) ^b
Group B	4.2 (2/47) ^a	2.29 (1/45) ^{ab}	2.3 (1/44) ^b	8.5 (4/47) ^b
Group C	6.5 (3/46) ^a	7 (3/43) ^{ab}	17.5 (7/40) ^a	28.3 (13/46) ^a
Group D	6.4 (3/47) ^a	11.4 (5/44) ^a	17.9 (7/39) ^a	32 (15/47) ^a

^{a,b} Comparison differences in mortality between groups at different periods (i.e., within column). Values with the same superscript are not significantly different from each other ($p \geq 0.05$), but they differ significantly ($p < 0.05$) from values with a different superscript.

3.5. Lung Lesion Score (LLS) and Pleurisy Lesion Score (PLS)

LLS and PLS values (mean $\pm$ SD) are shown in Table 6. The results of the scoring of the lungs at the piglets' slaughter indicated that vaccinated groups (A and B) had significantly fewer lesions and pleurisy in comparison to non-vaccinated groups (C and D) ($p < 0.05$). However, no significant differences were found between vaccinated groups A and B, as well as between the non-vaccinated groups (C and D).

Table 6. Lung lesion score (LLS) and pleurisy lesion score (PLS) at slaughter (Mean $\pm$ SD).

Groups	LLS at Slaughter	PLS at Slaughter
Group A	7.76 $\pm$ 2.89 ^b	0.06 $\pm$ 0.25 ^b
Group B	7.57 $\pm$ 2.99 ^b	0.06 $\pm$ 0.32 ^b
Group C	11.44 $\pm$ 6.43 ^a	0.46 $\pm$ 0.75 ^a
Group D	11.84 $\pm$ 7.07 ^a	0.45 $\pm$ 0.77 ^a

^{a,b} Comparison of LLS and PLS at slaughter between groups (i.e., within the column). Values with the same superscript are not significantly different from each other ($p \geq 0.05$), but they differ significantly ($p < 0.05$) from values with a different superscript.

3.6. Study Power Results

Using as an input for the G*Power software each one of the measured study variables, the study achieves more than 95% power. The protocol of the power analyses and the plots showing

the study power as a function of the sample size and effect size (a quantitative measure of the magnitude of the experimental effect) are presented in Supplementary File S1.

4. Discussion

The results of this study are important indications that the vaccination of piglets with the Porcilis® PRRS vaccine via the intradermal route had a positive effect on their protection against PRRSV viremia, equally of a vaccine with the intramuscular application. Based on the qRT-PCR results obtained by testing the serum samples, we noticed that the percentage of viremic animals in group A was lower at 10 weeks in comparison to other groups, as well as the percentage of vaccinated groups in comparison to non-vaccinated groups at 7, 10, and 13 weeks. However, significant differences were noticed only in 10 weeks in the two vaccinated groups (A and B). The viremia titer estimation based on the Ct values revealed higher PRRSV loads in non-vaccinated groups (C and D) at 7, 10, and 13 weeks. Our results agree with previous studies, which reported that the ID vaccination with the PRRSV MLV vaccine does not affect the degree of virological protection, providing similar or even better protection compared with an IM vaccination [24,25,32]. However, these studies are reported on the vaccination of piglets at the weaning age, while the novelty of our study is the first report of ID vaccination results in younger piglets (suckling piglets at 14 days of age).

Generally, PRRSV MLV vaccines prove to be partially effective in reducing the clinical signs, viremia, and virus shedding [40]. Moreover, MLV vaccines offer consistently efficient protection against a homologous challenge, while their provided protection against a heterologous challenge is neither as consistent nor as effective [1,40,41]. The field PRRSV strain in this trial could be considered heterologous to the vaccine virus (strain DV), as the ORF 5 sequence comparison between them revealed a 90.7% sequence identity. A homology less or equal to 97–98% can be considered as a different PRRSV strain, although no defined cut-off was published [42]. Even though genetic differences between the field strain and the vaccine

DV strain were evidenced, the tested PRRSV MLV vaccine provided a partial reduction in viremia in vaccinated groups (groups A and B) at 10 weeks in comparison to 7 weeks, possibly due to the infection of pigs by the field strain after the vaccine's administration. Published controlled studies have estimated the length of viremia after a PRRSV vaccination with MLV vaccines to be 29 days on average (range 10–42 days) [25,28,43,44,45,46]. However, future studies are recommended to better understand the infection dynamics and the induced protection by vaccination.

Based on the obtained ELISA and qRT-PCR results, indications were provided that the PRRSV MLV vaccination of piglets by the ID or IM route induces an important seroconversion in 4 and 7 weeks (2–5 weeks post-vaccination) in comparison to non-vaccinated groups. In our trial, nearly 100% of pigs in vaccinated groups (groups A and B) were seropositive at week 4 (2 weeks after vaccination) while the percentage of seropositive pigs dropped significantly at week 7 and by weeks 10 and 13; all pigs in the vaccinated groups become seropositive. The possible explanation is that the vaccinated pigs were infected with a field virus, and therefore seroconverted in weeks 10 and 13. Moreover, the level of PRRSV-specific antibodies at the end of the finishing stage (21 weeks) is significantly lower in group A compared to those of other groups. However, these findings do not provide clear evidence of whether the ID-vaccinated animals of group A had a more efficient protection due to the high antibody levels in the other groups. Based on previous studies [47] and our results, the hypothesis that the transmission rate was probably lower in the ID vaccinated group A than in non-vaccinated piglets, as well as the duration of infectiousness, can be supported. Consequently, future studies are required to investigate if the ID vaccination of piglets at 14 days of age could considerably decrease the spread of PRRSV and fulfil the demands of farmers for a more efficient and cost-effective production, based on the decreased probability of piglets being infected or vaccinated piglets' spread of the virus once they are infected [48].

The protective efficacy of a PRRS MLV vaccine is of a great economic importance due to the improvement of the health status and growth performance of vaccinated pigs [1,7,13]. Moreover, the effectiveness of a PRRS MLV vaccine must be accompanied by the absence of adverse reactions. The results of the current study proved the safety of the tested commercial ID PRRSV MLV vaccine, as no significant local and systemic reactions were noticed in vaccinated piglets until 14 days post-vaccination. Our results agree with previous studies with PRRSV MLV vaccines in gilts and weaning piglets under field conditions [24,25,28,49]. However, the literature on the safety of an ID PRRSV vaccination in suckling piglets is very limited. Previous studies with an ID vaccination in suckling piglets against other respiratory pathogens (*M. hyo*, PCV2) reported less vaccination-associated behavior changes in ID-vaccinated suckling piglets in comparison to IM-vaccinated suckling piglets, such as more active behavior and suckling activity, fewer flight reactions at the time of vaccination and less number, duration, and intensity of vocalizations [50,51]. The weakness of our study is that even if we noticed generally similar clinical observations, behavior changes in ID- and IM-vaccinated suckling piglets were not recorded and analyzed.

PRRS is characterized by a reduction in the growth performance parameters (e.g., BW and ADG) [52,53,54]. In the current study, only the ID vaccination has a significantly positive impact on the BW at the piglets' slaughter in comparison to non-vaccinated group C, while both the ID and the IM vaccination had a significantly positive impact on the ADG in comparison to both non-vaccinated groups C and D. Previous studies have reported that a PRRSV MLV vaccination has beneficial effects on the growth performance and decreased frequency of the clinical signs in piglets exposed to PRRSV [55,56,57]. The differences in the BW and ADG observed among groups in our study were not as remarkable as previous studies had showcased [58,59,60]. This could be attributed to the short time between the vaccination and the

occurrence of the natural infection, which did not allow for the improvement of the growth performance in vaccinated animals [48].

Mortality rates appear to be the most usually affected parameters by a PRRSV infection in piglets [5]. In our trial, the mortality rate was significantly lower in vaccinated piglets in comparison to non-vaccinated piglets at the finishing stage; the same applied to the total mortality percentage. In addition, the mortality rate was significantly lower in the vaccinated groups (A and B) than in non-vaccinated group D at the growing stage. The beneficial effects of a PRRSV vaccination on the mortality rate are correlated with our results from lung scoring. The lung scoring at the piglets' slaughter indicated that vaccinated groups (A and B) had significantly less lung and pleurisy lesions score in comparison to non-vaccinated groups. However, no significant differences were shown between vaccinated groups A and B and between the non-vaccinated groups. Our results indicate that both the ID and IM vaccination of piglets have beneficial effects on the health status of finishing pigs, reducing the LLS and PLS parameters, mainly due to secondary bacterial co-infections.

5. Conclusions

Previous studies showed that an ID vaccination against PRRSV has at least a similar immunological effect compared to the IM route, providing a similar protective immunity compared with an IM vaccination (Martelli et al. 2007, 2009; Jiang et al. 2021). Moreover, these studies included trials of the vaccination of piglets at the weaning age under experimental conditions. Therefore, the novelty of our study is the first report of an ID vaccination on suckling piglets at the specific age of 14 days of age and, most importantly, under field conditions. Even though the ID vaccination of piglets against PRRSV is a common practice to the best of our knowledge, there are no published field studies investigating the effect of an ID vaccination of suckling piglets against PRRSV on the growth performance parameters up to the finishing stage.

In conclusion, this study demonstrated the safety of the ID vaccination of suckling piglets with a PRRSV MLV vaccine at 2 weeks of age under field conditions. No significant local and systemic reactions were noticed in vaccinated piglets until 14 days post-vaccination. However, our study is the first report of an ID vaccination in suckling piglets at 14 days of age. Our study indicates that both the ID and IM vaccination of piglets decreased the mortality rate, as well as the lung and pleurisy lesions score in vaccinated groups at the finishing stage. However, the ID vaccination has a positive impact on the BW at the piglets' slaughter, while both the ID and IM vaccination had a positive impact on the ADG.

Our results are in agreement with previous studies and provide important evidence to showcase that an ID vaccination with the PRRSV MLV vaccine does not affect the degree of virological protection, providing a similar protection compared with the IM vaccination. The aim of our field trial focused on pointing out that “vaccine protection” is not necessarily equivalent to infection avoidance. Their protective action is also associated with (i) minimizing the duration of viremia and the viral titers in the bloodstream, (ii) limiting the shedding of the virus, thus minimizing the spread to naïve animals, and (iii) minimizing the clinical impact. Even if the outcome (mortality or lung lesion scores) was not similar between IM and ID vaccinated animals, the differences in viremia are important, as they can potentially affect the shedding and infection of other animals within the farm. Additionally, as the vaccine used is attenuated, protection does not rely only on circulating antibodies, as other cell-mediated mechanisms are involved. However, such investigations or the further study of the differences following the booster immunizations of animal groups are beyond the aim of the present work.

Supplementary Materials

The following are available online at
<https://www.mdpi.com/article/10.3390/ani13010061/s1>.

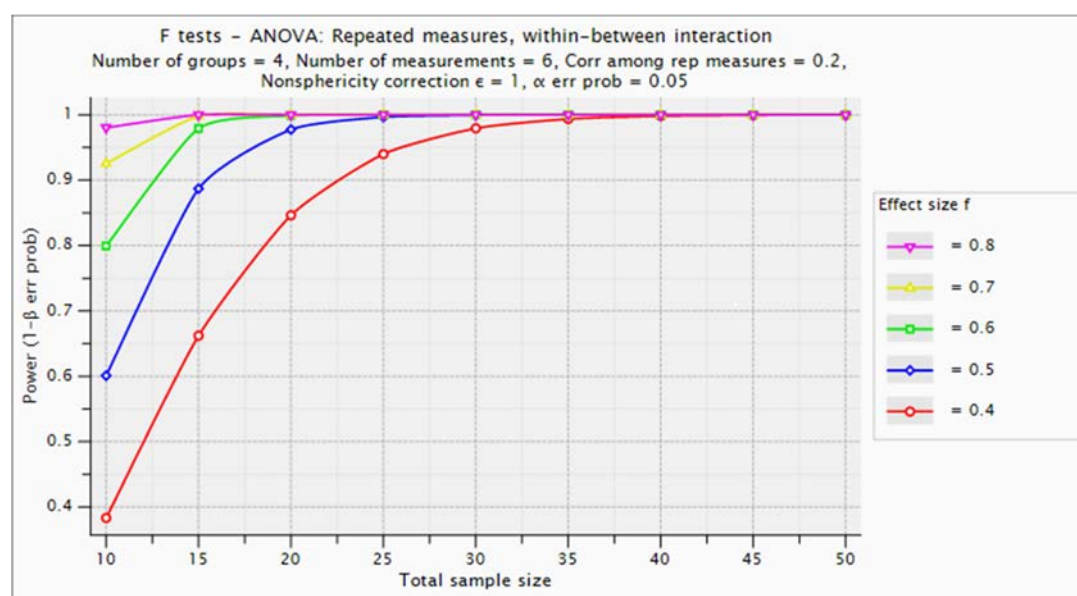
A) ELISA - qRT-PCR

F tests - ANOVA: Repeated measures, within-between interaction

Analysis: A priori: Compute required sample size

Input: Effect size f = 0.5
 α err prob = 0.05
Power ($1-\beta$ err prob) = 0.95
Number of groups = 4
Number of measurements = 6
Corr among rep measures = 0.2
Nonsphericity correction ϵ = 1

Output: Noncentrality parameter λ = 37.5000000
Critical F = 1.7932218
Numerator df = 15.0000000
Denominator df = 80.0000000
Total sample size = 20
Actual power = 0.9773699



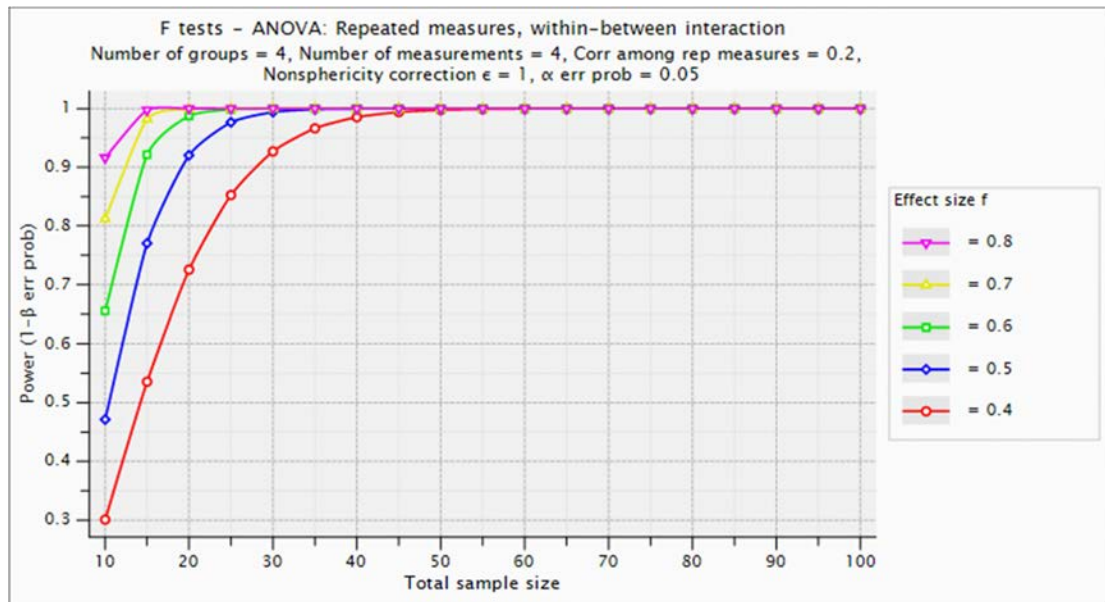
B) BW, Age at slaughter, ADG, Mortality

F tests - ANOVA: Repeated measures, within-between interaction

Analysis: A priori: Compute required sample size

Input: Effect size f = 0.5
 α err prob = 0.05
Power ($1-\beta$ err prob) = 0.95
Number of groups = 4
Number of measurements = 4
Corr among rep measures = 0.2
Nonsphericity correction ϵ = 1

Output: Noncentrality parameter λ = 30.0000000
Critical F = 2.0400981
Numerator df = 9.0000000
Denominator df = 60.0000000
Total sample size = 24
Actual power = 0.9697843



C) Lung score

F tests - ANOVA: Fixed effects, omnibus, one-way

Analysis: A priori: Compute required sample size

Input: Effect size f = 0.75

α err prob = 0.05

Power ($1-\beta$ err prob) = 0.95

Number of groups = 4

Output: Noncentrality parameter λ = 20.2500000

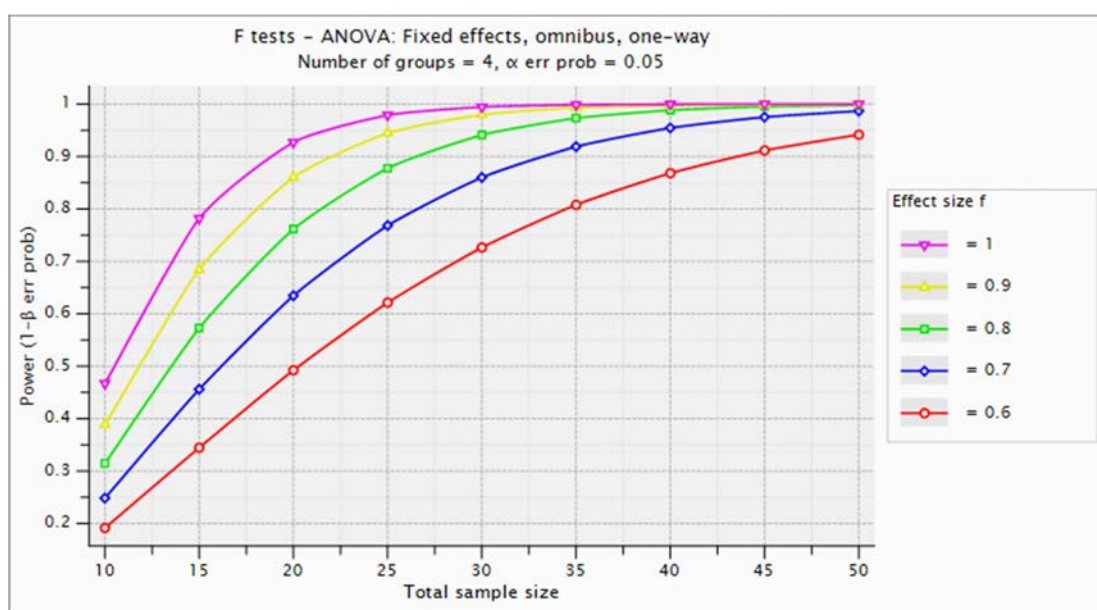
Critical F = 2.9011196

Numerator df = 3

Denominator df = 32

Total sample size = 36

Actual power = 0.9578945



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Institutional Review Board Statement

All animal procedures regarding animal care and use were approved by the Ethical Committee of the Faculty of Veterinary Medicine, School of Health Sciences, University of Thessaly (Approval number: 98/19-12-2019). The owner of the farm where the experimentation took place was informed in detail and provided written consent for the use of animals on his farm in the present study.

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CHAPTER III

Investigation of Fas (APO-1)-Related Apoptosis in Piglets Intradermally or Intramuscularly Vaccinated with a Commercial PRRSV MLV

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) remains a major cause of significant economic loss in the swine industry (44). PRRSV causes reproductive failure in the breeding herd and respiratory disease and growth retardation in the pigs (65). Vaccination is considered as a crucial measure for the prevention and control of PRRSV infection. It is also the most cost-effective strategy for all sizes of pig farms compared with other control strategies (28,45).

Intradermal (ID) vaccination has the advantage of targeting antigen-presenting cells in the epidermis near skin-draining lymph nodes (46,57). Many studies have already proved the safety and efficacy of ID vaccination against major pathogens in swine, such as PRRSV (2,33,34,50), porcine circovirus 2 (PCV2) (49), Aujeszky's disease virus (12,61), and *Mycoplasma hyopneumoniae* (*M. hyo*) (4,55). Combined with needle-free administration, ID vaccination is also more animal friendly and prevents accidental transmission of pathogens caused by reusing needles as well as muscle damage by broken needles in the muscle. Previous studies demonstrated that route of administration of the vaccine does not affect the degree of virological protection, as pigs intradermally (ID) vaccinated did not show any differences in terms of viral load in their blood and clinical signs when compared to animals intramuscularly (IM) vaccinated (33,34). Previous reports confirmed that a PRRSV modified live vaccine (MLV) administered via the intradermal route can efficiently induce a protective response in pigs subsequently exposed to a genetically diverse PRRSV isolate even when applied in field conditions (33). This means that vaccine administration via either the intradermal or the intramuscular route reduces the viral titers of PRRSV in the blood and in BAL fluid, can confer some degree of virological cross-protection to heterologous isolates of PRRSV (26). Also, a recent study suggested that a European type 1 (PRRSV-1) PRRSV MLV administered by either IM or ID can provide partial heterologous protection after challenge with a highly pathogenic

PRRSV type 2 (PRRSV-2) strain, either alone or combined with PRRSV-1, as demonstrated by less lung tissue lesions and reduced viremia (31). In addition, needle-free applications have demonstrated some advantages such as safety for the executor, painlessness for the pig, absence of histological lesions, and no iatrogenic transmission of pathogens (33). It has been demonstrated that 28 hours following vaccination, IM vaccinated piglets against PCV2 had lower levels of blood C-reactive protein and blood haptoglobin compared to IM vaccinated ones (56). Another advantage is that ID vaccine administration might represent an alternative to enhance the efficacy of vaccination against PRRSV, as it induced lower IL-10 levels after vaccination and more IFN- γ -SC after the experimental challenge of animals with a heterologous viral strain alone or even combined with a homologous strain infection (31). Notable welfare associated advantages of ID vaccination versus IM vaccination of piglets have been demonstrated in recent studies. ID vaccination against *M. hyo* (14) and PCV2 (56) resulted in fewer post-vaccination behavior and activity changes in suckling piglets compared to intramuscular vaccination.

Pulmonary alveolar and/or intravascular macrophages are known as the major target cells for PRRSV in the lungs (58). The studies on PRRSV infection and replication in host alveolar macrophages continue to give conflicting results as to its effect on innate cell activation, antigen presentation, and the triggering of acquired immunity (21,23,25,27,59,60). It has been widely demonstrated that PRRSV causes apoptosis in different organs (lungs, testes, lymph nodes, and thymus) as well as in cell lines (5,6,11,20,24,48,51,52). On a morphological basis, the apoptotic cells are assumed to be alveolar and pulmonary intravascular macrophages and mononuclear cells in the alveolar septa. Double labelling experiments indicate that most apoptotic cells are non-infected (48,52). An infection of the lungs with PRRSV has some peculiar cellular features that may be attributed to apoptosis. Firstly, it is characterized by changes in the population of bronchoalveolar monocytes/macrophages, consisting of a reduction of the population of well-

differentiated macrophages and an influx of new monocytes (22). In general, apoptotic pathways can be divided into two nonexclusive signalling cascades: extrinsic pathway and intrinsic pathway (7). Extrinsic, or “death” receptor-mediated apoptosis is initiated when a soluble ligand (such as tumor necrosis factor, TNF; Fas ligand, FasL; or TNF-related apoptosis-inducing ligand, TRAIL) binds to specific receptors on the cell surface (1,38).

The cell-surface Fas receptor, also termed APO-1 or CD95, is a member of the tumor necrosis factor (TNF) and nerve growth factor (NGF) family of receptors (39,47). Fas expression is observed in a variety of immune and non-immune cells (62). Previous studies have covered the mechanisms underlying cell death induced by Fas/Fas ligand (FasL) interaction (1,19,32,43).

The aim of the present study was to investigate the Fas (APO-1)-related apoptosis in piglets following intradermal (ID) and intramuscular (IM) vaccination with a PRRSV MLV in comparison with non-vaccinated piglets.

2. Material & Methods

2.1 Ethics

Throughout this clinical study every procedure was performed according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching as well as the Code of Practice for the Conduct of Clinical trials for Veterinary Medical Products, all animals were housed and handled in compliance with National and European animal Welfare requirements (8,9,10,42). All procedures and animal use in this study were approved by the Ethical Committee of the Faculty of Veterinary Medicine, School of Health Sciences, University of Thessaly (Approval number: 98/19.12.2019).

2.2 Farm

General requirements for the selection of the farm were the presence of a PRRSV laboratory- confirmed infection and good farm management practice (i.e., accurate and up-to-date administration and all-in/all-out system).

The present study was carried out in a farrow-to-finish commercial pig farm (commercial hybrids of Large White x Landrace). The capacity of the farm was 150 sows under production, located in Central Greece. A grandparent nucleus of 10 sows was kept in the farm for producing its own gilts. The farm facilities included farrowing houses, flat-deck units, growing and finishing houses, one mating-pregnancy (dry period) stable with individual stalls (35th – 105th day of pregnancy), breeding stock house of group housing (>35th day of pregnancy), breeding stock house of group housing for non-inseminated gilts, a feed mill, and an artificial insemination (AI) laboratory. The herd practiced a 1-week batch production system. The weaning piglets were allotted equally according to the body weight and sex at random at flat-deck batteries for piglets in a climate-controlled post-weaning stable. Weaned sows were housed in individual stalls, and they were inseminated twice with fresh semen from the same boar. Semen collection, dilution, and storage were performed on the farm (system “Do-it-yourself AI”). At 30-35 days of gestation, sows were moved to group housing rooms where they were housed in 10 animal groups. Sows were fed individually using separate feeding stalls. Gilts were also housed individually during the first month of gestation.

The routine vaccination program of breeding stock included vaccinations against PRRSV, Aujeszky's disease virus, swine influenza virus, porcine parvovirus, *Erysipelothrix rhusiopathiae*, *Escherichia coli* and *Clostridium perfringens*. Gilts were vaccinated at age of 180th and 210th days and the sow population followed a 6-60 vaccination plan against PRRSV with the commercial vaccine (Porcilis® PRRS, MSD Animal Health). The weaned piglets were vaccinated against *M.*

hyo and PCV2 at 21 days of age. All breeding females were treated with a single ivermectin injection 14 days prior farrowing; boars were treated twice a year.

Feed provided to the animals was self-prepared meal, based on a corn/barley/wheat-soya combination, depending on the season. Breeding animals received differentiated meal during gestation and lactation, based on the needs of the animals for each production stage.

Animals had *ad libitum* access to drinking water. There was one drinking nipple per sow during lactation and one nipple in each crate during gestation. Housing facilities were equipped with a fully automated temperature and humidity control system, as well as an automated feeding system.

2.3 Farm history – pre-trial period

One month before the beginning of the trial, blood samples were collected from the breeding stock (12 total blood samples from gilts, sows in the dry period, and in lactation), as well as from suckling, weaning, growing, and finishing pigs (8 blood samples at age of 2, 4, 7, 10, 13, 17, and 21 weeks). The presence of PRRSV infection was investigated by real-time reverse transcription (RT)-PCR (qRT-PCR) and the wild type PRRSV strain circulating in the study farm was characterized by complete ORF5 (Gp5) gene sequencing and phylogenetic analysis, as described below under the section “Laboratory examinations”.

2.4 Animals

In total 104 suckling piglets of 2 weeks of age were included in the study and were divided into 4 groups (Table 1) and two replicates, including 13 piglets per group (2 replicates x 13 piglets x 4 = 104 piglets). Only healthy suckling piglets of 2 weeks of age were included. Diseased piglets, piglets significantly underweight, runts, and animals with abnormalities (e.g., hernias) were excluded. All piglets were identified with two unique numbered ear tags. Piglets belonging to all groups were mixed and housed as usual.

2.5 Study design

2.5.1 Experimental material

Porcilis® PRRS (MSD Animal Health) was the commercial vaccine used in the study for the immunization of piglets against PRRSV. The vaccine was administered both via intramuscular and intradermal ways as stated in product SPC. It is a live attenuated lyophilized-vaccine containing per dose of 2 ml (intramuscular application) or 0.2 ml (intradermal application) of reconstituted vaccine: Lyophilisate (active substance) – Live attenuated PRRSV strain DV: $10^{4.0} - 10^{6.3}$ TCID₅₀ (50% tissue culture infective dose), Solvent (Diluvac Forte® adjuvant) – dl- α tocopheryl acetate: 75 mg/ml. In control groups, only Diluvac Forte®, containing dl- α tocopheryl acetate was administered.

2.5.2 Experimental groups, dosage, and administration

The following four groups were created: Group A – Porcilis PRRS ID, Group B – Porcilis PRRS IM, Group C – Diluvac ID and Group D – Diluvac IM. Piglets belonging to the groups A and B were vaccinated at 2 weeks of age with 1 dose of Porcilis® PRRS, diluted in 0.2 ml of Diluvac Forte® and injected intradermally (ID), or diluted in 2 ml of Diluvac Forte® and injected intramuscularly (IM). Piglets belonging to groups C and D were not vaccinated against PRRSV. Only 1 dose of the recommended volume of adjuvant (Diluvac Forte®) was administered to each animal belonging to both latter groups. Specifically, 0.2 ml for ID negative controls (group C) and 2 ml for IM negative controls (group D).

Per treatment group, an IDAL (IntraDermal Application of Liquids) vaccinator was used for needle-free intradermal vaccination of ID groups. For the vaccination of the IM groups an automatic syringe, with a fixed 2 ml volume, was used. A new, clean needle (size 86, approx. 0.9x13 mm) was used for each IM trial group.

All other treatments of the growing-fattening pigs (preventive oral or injectable treatments and vaccinations) were recorded for each treatment and control group.

2.5.3 Samplings

During the trial, blood samples were collected from 3 same ear-tagged pigs of each group for two replicates, at the age of 4, 7, and 10 weeks, as shown in Figure 1.

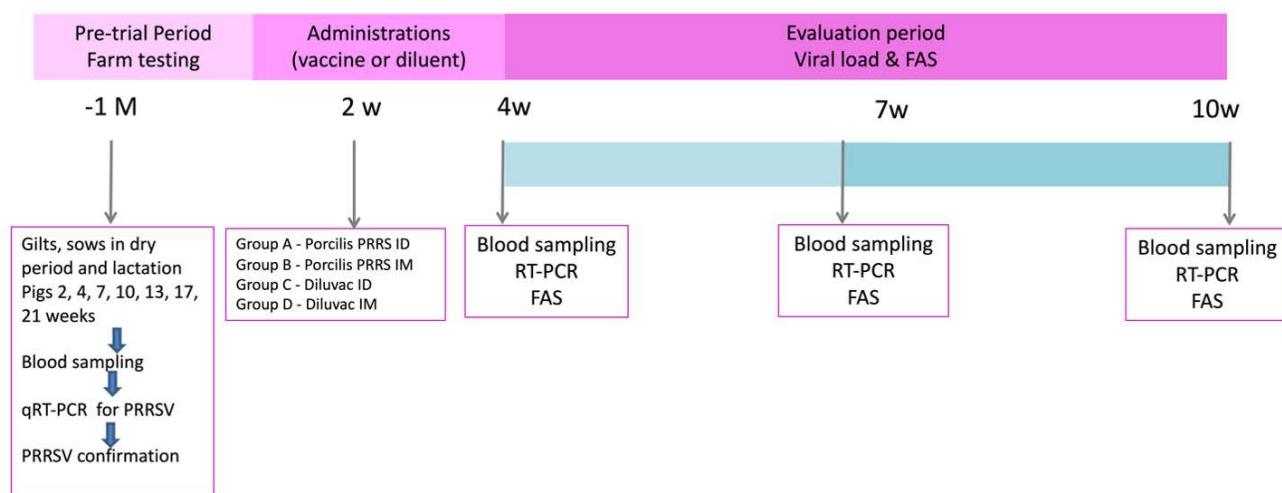


Figure 1. An overview of experimental design, samplings and tests performed.

2.6 Laboratory examinations

Blood serum samples obtained both from the pre-trial period and the trial (investigation) period underwent nucleic acid extraction using the PureLink®Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA). Extracts were tested for PRRSV genome, using a previously described TaqMan probe-based qRT-PCR (30). Reactions were carried out on a CFX96® Real-Time System (Bio-Rad Laboratories, Hercules, CA). The obtained cycle threshold (Ct) values were used as viral load estimates regarding the samples obtained during the trial period.

In addition, the wild type PRRSV strain circulating in the study farm was characterized by complete ORF5 (Gp5) gene sequencing and phylogenetic analysis, including sequence comparison with the vaccine strain used in the study. Briefly, a qRT-PCR-positive RNA extract was reverse transcribed into cDNA using random hexamers. The viral ORF5 sequence was amplified by PCR, employing the primers Gp5-SeqUp (5'-

ATGAGGTGGGCYACAACCATYGC-3') and Gp5-SeqDo (5'-AGGGCRTATATCATTATRGGTGTGTATGT-3'). Sanger sequencing of the purified product was performed on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). The obtained ORF5 sequence was assembled and imported into MEGA 6 software (53). Additional homologous sequences of representative European type viral strains were retrieved from GenBank, including the sequence of the strain contained in the vaccine used (strain DV; Acc. no. KF991509), and were included in the dataset. The sequences were aligned by MUSCLE. The best-fitting nucleotide substitution model was determined based on the lowest Bayesian information criterion (BIC) score. A maximum likelihood (ML) tree was constructed using the Kimura 2-parameter model with gamma-distributed rates across sites (K2+Γ) and the nearest-neighbor interchange (NNI) tree searching strategy. The reliability of the hypothesis was assessed using bootstrap resampling with 1000 replications. Pairwise genetic distance calculation was performed by MEGA 6.

Sera were also tested for sFas levels using a commercially available ELISA Kit (Pig Factor-related Apoptosis, ELISA kit Porcine FAS/APO-1, Cusabio) following the manufacturer's instructions.

2.7 Statistical analysis

Data were expressed as mean $\pm$ standard deviation. Comparisons were performed using Kruskal-Wallis's test while the Mann-Whitney U test was used for post hoc/multiple comparisons. Comparisons between more than two measurements of the same group during the time were performed using Friedman's test and Wilcoxon's signed-rank test as a post hoc test. All tests were two-sided. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed in IBM SPSS 21 (IBM Corp, Armonk, NY). Correlation between viral load as determined by qRT-PCR and the Fas levels in the blood was estimated by Spearman's correlation coefficient (ρ), employing the commercial MedCal 9.2 software

(MedCalc Software, Mariakerke, Belgium). Statement of significance was based on $P \leq 0.05$. The strength of the relationship was evaluated as follows: $\rho \leq 0.35$ - weak correlations, 0.36 to 0.67 - moderate correlations, and 0.68 to 1.0 - strong correlations (55).

3. Results

3.1 Virological testing

During the pre-trial period, the presence of PRRSV infection was confirmed by qRT-PCR in blood serum samples. At an age of 4, 7, 10, and 13 weeks 100% of the tested pigs were qRT-PCR -positive. The full length ORF5 sequence of the wild type PRRSV strain detected in the study farm during the pre-trial period was successfully determined. Comparison of the obtained sequence with respective sequences of other European genotype strains available in GenBank, revealed nucleotide sequence identity of 90.7% to the strain DV, i.e. the strain included in the MLV vaccine used in the study (acc. no. KF991509) and of 90.8% to “Lelystad virus”, i.e. the prototype European genotype strain (acc. no. M96262) (Figure 2).

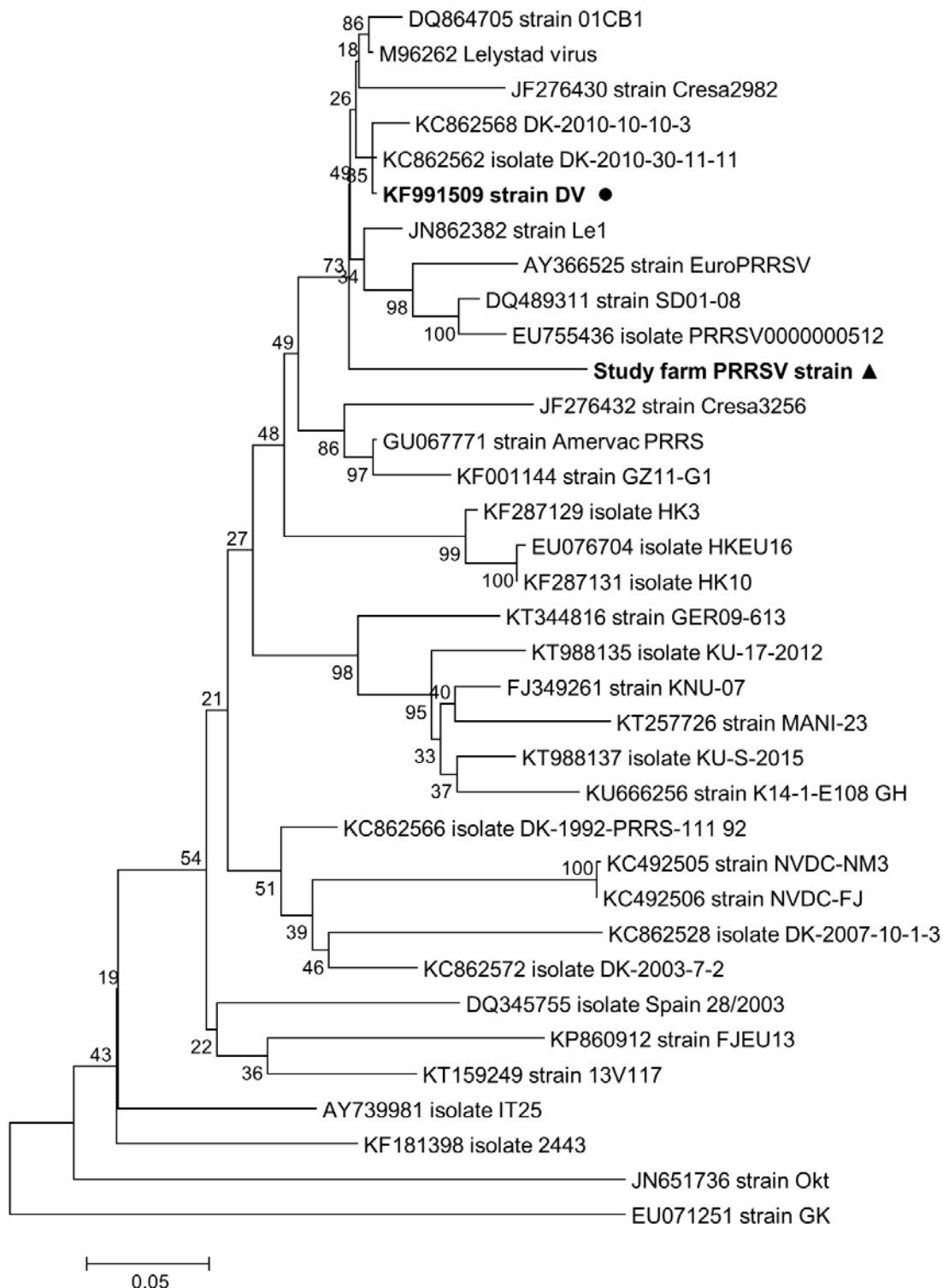


Figure 2. Phylogenetic tree inferred with maximum likelihood analysis based on complete ORF5 (Gp5) sequences of selected European type viral strains. Each taxon label includes GenBank accession number, and isolate/strain name. The Kimura 2-parameter model with

gamma-distributed rates across sites (K2+Γ) was selected as the best fitting nucleotide substitution model for this dataset. The tree is rooted at the midpoint and the numbers indicated on the branches are nonparametric bootstrap probabilities generated from 1000 iterations. The scale bar indicates nucleotide substitutions per position. The sequence obtained from the wild type virus circulating in the study farm is indicated with a triangle and the sequence of the DV vaccine strain is indicated with a circle

The results of qRT-PCR analysis in the blood samples obtained during the study period are shown in Table 1. Ct values are presented in Figure 3. Samples obtained at 4 weeks of age were negative in all groups. At 7 weeks only group A was negative and at 10 weeks all groups were positive. However, a narrower Ct value range and a slightly lower mean Ct value were observed in group A compared to group B at 10 weeks (Figure 3).

Table 1. Results of qRT-PCR in serum samples of the studied animals at ages of 4, 7, and 10 weeks.

Replicates	Time of blood sampling		
Replicate 1	4 weeks	7 weeks	10 weeks
Group A (Porcilis PRRS ID)	PRRSV-negative	PRRSV-negative	PRRSV-positive (3/3)
Group B (Porcilis PRRS IM)	PRRSV-negative	PRRSV-negative	PRRSV-positive (3/3)
Group C (Diluvac ID)	PRRSV-negative	PRRSV-positive (2/3)	PRRSV-positive (3/3)
Group D (Diluvac IM)	PRRSV-negative	PRRSV-positive (2/3)	PRRSV-positive (3/3)
Replicate 2	4 weeks	7 weeks	10 weeks
Group A (Porcilis PRRS ID)	PRRSV-negative	PRRSV-negative	PRRSV-positive (3/3)
Group B (Porcilis PRRS IM)	PRRSV-negative	PRRSV-positive (2/3)	PRRSV-positive (3/3)
Group C (Diluvac ID)	PRRSV-negative	PRRSV-positive (3/3)	PRRSV-positive (3/3)
Group D (Diluvac IM)	PRRSV-negative	PRRSV-positive (3/3)	PRRSV-positive (3/3)

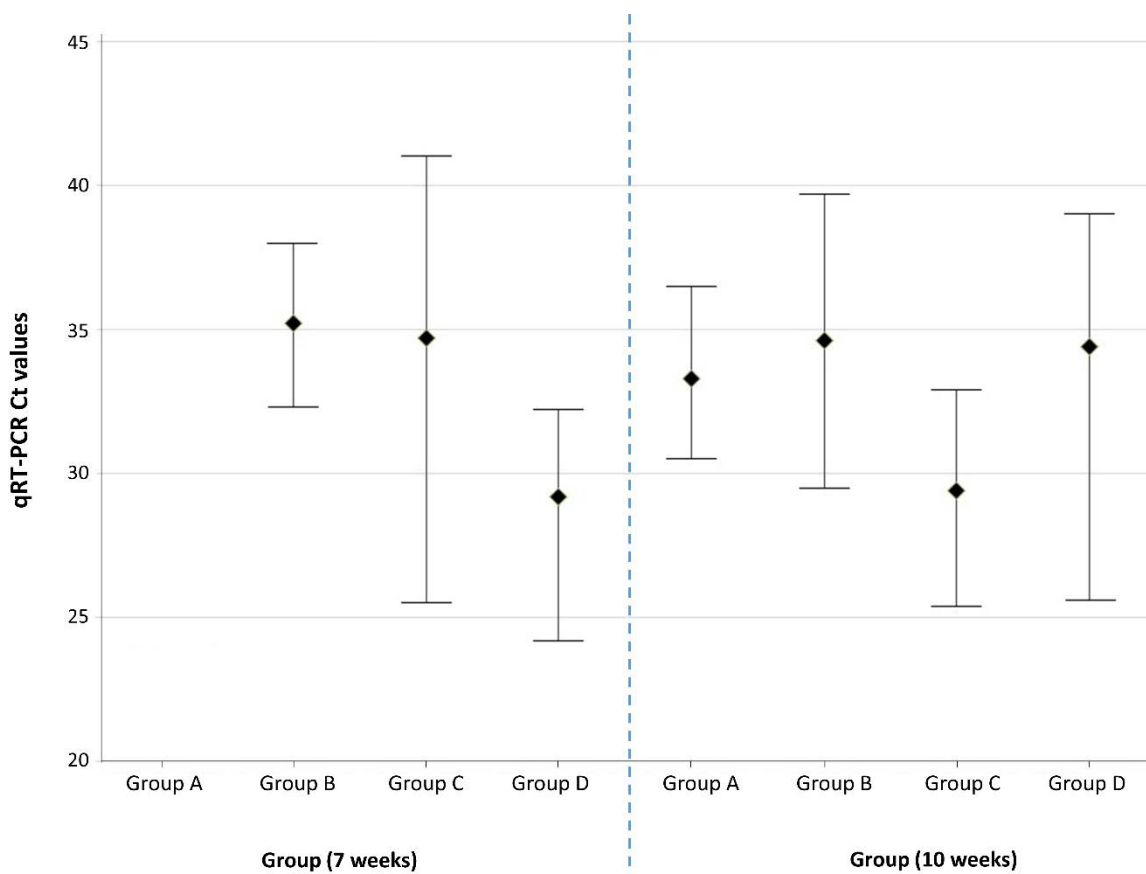


Figure 3. Ct values of qRT-PCR in serum samples at ages of 7 and 10 weeks of PRRS ID and IM vaccinated piglets in comparison with unvaccinated piglets

3.2 *Fas*

The results for *Fas* (pg/ml) are shown in Table 2 and Figure 4. No differences in s*Fas* levels were observed in groups A and B throughout the study. Levels of s*Fas* increased in group C during time (4 vs 7 weeks- $p=0.028$, 4 vs 10 weeks- $p=0.028$, 7 vs 10 weeks- $p=0.046$) and D (7 vs 10 weeks- $p=0.028$). Significant differences among groups were noticed only at 10 weeks (A vs C- $p=0.010$, A vs D- $p=0.055$, B vs C- $p=0.010$, B vs D- $p=0.037$).

Table 2. Results of Fas (APO-1) levels (pg/ml) in serum samples at ages of 4, 7 and 10 weeks.

Group	Time (weeks)	Fas (pg/ml) Mean ± SD	Significance of differences in the same group during time	Significance of differences among groups	
Group A (Porcilis PRRS ID)	4	249.39 ± 72.03	NS (non-significant)	4 weeks: NS	
	7	367.76 ± 88.37		7 weeks: NS	
	10	293.93 ± 49.88		10 weeks: A vs B: NS A vs C: p=0.010 A vs D: p = 0.055 B vs C: p=0.010 B vs D: p=0.037 C vs D: NS	
Group B (Porcilis PRRS IM)	4	260.23 ± 71.23	NS		
	7	270.13 ± 55.19			
	10	281.72 ± 56.88			
Group C (Diluvac ID)	4	199.24 ± 32.07	4 weeks vs 7 weeks p=0.028		A vs B: NS A vs C: p=0.010
	7	329.37 ± 35.42	4 weeks vs 10 weeks p=0.028		A vs D: p = 0.055 B vs C: p=0.010
	10	390.01 ± 52.35	7 weeks vs 10 weeks p=0.046		B vs D: p=0.037 C vs D: NS
Group D (Diluvac IM)	4	275.96 ± 14.70	7 weeks vs 10 weeks p=0.028		
	7	271.87 ± 76.77			
	10	370.67 ± 66.06			

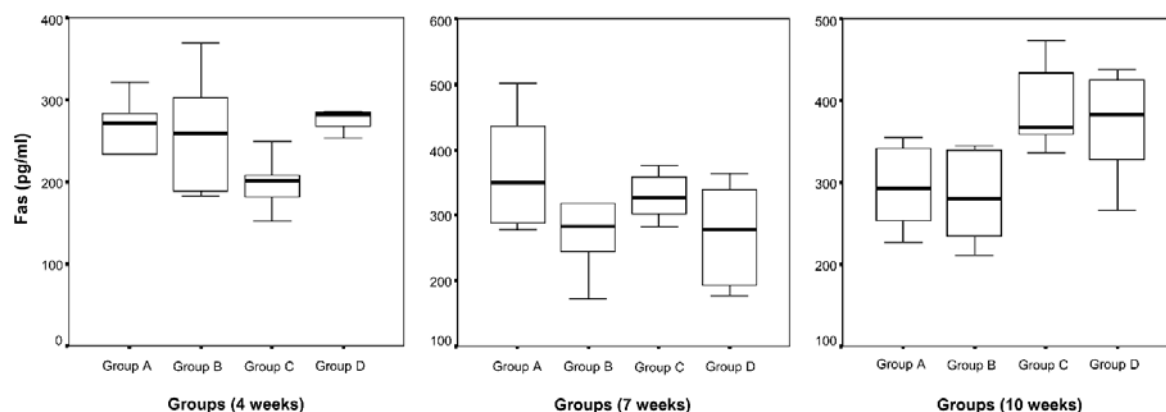


Figure 4. Fas levels (pg/ml) in all groups at age of 4, 7, and 10 weeks of PRRS ID and IM vaccinated piglets in comparison with unvaccinated piglets

3.3 Correlation between PRRSV viral load and Fas

Data analysis revealed a statistically significant positive and moderate correlation between PRRSV viral load and Fas level ($\rho=0.639$, $p<0.0001$).

4. Discussion

Previous studies have determined the regulatory roles of PRRSV in apoptosis that is important in the pathogenesis of PRRSV (16,18,24,51,52). In our study, increased serum sFas levels in unvaccinated piglets were recorded, revealing apoptotic suppression compared to vaccinated piglets. In the latter, vaccine-derived antibodies limit the infection and may attribute to the reduced Fas expression, suggesting a weak induction of lymphocyte-mediated cytotoxicity. Finally, higher sFas levels were observed with aging, possibly due to persistent PRRSV infection.

Virus-infected cells involved apoptosis in eliminating themselves to prevent viral replication, viral dissemination, or persistent infection (41). Thus, it is vital for viruses to disable host cell apoptosis at an early stage of the infectious cycle, thereby avoiding premature cell death and allowing viral replication. On the other hand, apoptosis at a late infection stage could

function promotively to virus spread by breaking down infected cells or killing uninfected cells of the immune system (15). Former studies demonstrated biphasic apoptosis during virus infection (17,64). In contrast with a typical acute viral infection resolving in 1-2 weeks by an effective clearance, PRRSV gives rise to a long-term infection, characterized by prolonged viremia and persistent infection. The immune response to PRRSV is partially ineffective, including a relatively low or altered activation of the inflammatory and innate response, which has an important role in accounting for the persistence of PRRSV infection and its low and retarded activation of acquired immune response (3,29,37,63). PRRSV-1 has been shown to possess a dichotomous apoptotic role in manipulating apoptosis, which is inhibited early but induced late in infection (6).

Fas/Fas ligand (FasL) demonstrated a major role in the induction of apoptosis in immune and bronchial epithelial cells (13,35,40). Fas/FasL interactions induce apoptosis of immune cells including T, B cells, and macrophages and cell death is often associated with activated stages of immune cells. Activation-induced cell death is a mechanism to inhibit excessive immune responses and to terminate ongoing immunity. The analysis of our results demonstrated for first time a significant positive and moderate correlation between PRRSV viral load and Fas levels. This finding supports that PRRSV interferes with the induction of cellular innate immune response, pathways of which are tied closely with that of apoptosis (36). This may explain why there is a delayed immune response in PRRSV-infected pigs and elucidation of the mechanisms of interference may provide clues into novel intervention strategies to combat PRRSV infection (36).

In conclusion, our study indicated increased serum sFas levels in unvaccinated piglets, which could reveal apoptotic suppression compared to vaccinated piglets. No significant differences were noticed for serum sFas levels between ID and IM vaccination.

Moreover, in the latter, vaccine-derived antibodies limit the infection and may attribute to the reduced Fas expression, suggesting a weak induction of lymphocyte-mediated cytotoxicity.

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CHAPTER IV

Angiotensin II Blood Serum Levels in Piglets, after Intra-Dermal or Intra-Muscular Vaccination against PRRSV

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) infection has a significant economic impact on pig production globally [1], due to reproductive failure in sows and respiratory disease in piglets and fattening pigs [2]. Vaccination remains the most crucial and cost-effective preventive tool in the fight to limit PRRS infection and its consequences [3,4]. Commercial PRRSV modified live virus (MLV) or killed virus (KV) vaccines are now available for use in sows or piglets and sows, in many countries worldwide. However, modern available vaccines do not offer effective prevention of viruses spreading within a herd [1,3]. Experimental challenge and field studies reported that PRRSV-MLVs create delayed but effective protection against wild-type PRRSV strains with genetic homology, while they provide only partial or no protection at all against heterologous strains [1,5,6].

Intramuscular (IM) vaccination, using needles, has usually been applied on pig farms; however, risks associated with needles have increased. Intradermal (ID) vaccination has the advantage, compared with IM administration, of easy access to dendritic cells at the site of injection, and targeting antigen-presenting cells in the epidermis near skin-draining lymph nodes [7,8]. This route of vaccine administration induces a faster and more direct response of the vaccinated pigs to the antigen-induced with the vaccine. ID vaccination in pigs has been reported to provide efficient protection of the pigs against infections with other pathogens, such as Aujeszky's disease [9], PRRSV [10,11,12,13,14], Porcine Circovirus 2 (PCV2) [15,16] and porcine enzootic pneumonia (*Mycoplasma hyopneumoniae*) [17,18]. ID vaccination is proposed as an alternative vaccination route to enhance vaccine safety, inducing lower IL-10 levels and more interferon- γ -secreting cells (IFN- γ -SC), reduction of PRRSV shedding within the herd and decreasing the potential iatrogenic transfer of pathogens between pigs by the use of needles [19,20]. In addition, ID vaccination offers animal welfare benefits, eliminates accidental

injuries in farm workers by needles, and reduces injection tissue damages caused by IM vaccine administration, which are quite prevalent in pigs in slaughterhouses [21].

A soluble glycosylated protein (molecular mass, 50–60 kDa), angiotensinogen is the only precursor of angiotensin (Ang) peptides as well as the sole known substrate for renin [22]. Angiotensinogen levels in blood circulation are relatively abundant (10^{-5} g or 10 μ g/mL; 200 nM), especially compared with Ang II or Ang-(1–7), and for this reason, a minimal amount (<0.1 mL) of serum or plasma is needed to measure angiotensinogen [22]. An ELISA method for angiotensinogen detection and quantification has been developed by Kobori and colleagues (2008) [23] and is available commercially and comprises antibodies directed against two distinct antigenic regions for the capture and detection of angiotensinogen. Ang II is a major factor of the renin-angiotensin system (RAS) for the regulation of blood pressure and cardiovascular homeostasis and is considered a systemic hormone, deriving from an enzymatic cascade [24]. Renin is firstly released from the juxtaglomerular cells of the kidneys into the blood circulation, where it proteolytically causes angiotensinogen to create the decapeptide Ang I. Ang I can be then cleaved by angiotensin-converting enzyme (ACE) and produces the octapeptide Ang II within the pulmonary circulation [25,26,27]. Apart from the circulating RAS, Ang II is produced in many other tissues and organs (heart, vasculature, kidney and brain), in the presence of angiotensinogen, renin and ACE, a fact that implies the paracrine and intracrine effects of Ang II [25,26,27,28,29].

Ang II performs a crucial part in the inflammatory response, cell proliferation, migration and apoptosis [30,31,32,33]. It is known that Ang II promotes apoptosis in human and rat alveolar epithelial cells and obstructs apoptosis in pulmonary endothelial cells during acute lung injury [34,35]. Many studies reported that PRRSV causes apoptosis, necrosis and necrosis-like-apoptosis in lungs and lymphoid organs leading to a detrimental effect on the immune system of the infected pig [36,37,38,39,40]. In addition, Ang II is a second important factor of the Fas

ligand (FasL) system in the lungs [41], while Fas/FasL is involved in PRRSV-induced apoptosis [42,43]. However, apoptosis could be induced in uninfected bystander cells by increased surface expression of FasL on surrounding cells caused by PRRSV infection, and as a result, these cells become sensitive to Fas-mediated apoptosis [42].

There are scarce published findings on the role of Ang II after IM or ID vaccination of swine against PRRSV. The ACE promotes the conversion of Ang I into vasoconstrictor Ang II and can be found in many tissues including vascular tissue [44]. ACE can promote vascular smooth muscle growth, capillary density and consumption of oxygen, affecting both sympathetic and neuromuscular transmission and having a hypertrophic effect on skeletal muscle, improving contractile function [45,46,47,48,49].

The goal of this study was to evaluate the concentration of Ang II in the serum of piglets following immunization against PRRSV with an MLV vaccine, through the ID and IM route, compared to non-vaccinated piglets.

2. Materials and Methods

2.1. Ethics

All procedures were performed according to the ethical standards and after receiving approval from the Animal Use Ethics Committee of Veterinary Faculty University of Thessaly (approval code: 98/19.12.2019).

2.2. Farm

The current trial was performed in a commercial farrow-to-finish pig farm of 150 sows (Topigs Norsvin hybrids of Large White × Landrace). The farm also maintained a grandparent nucleus of 10 sows in order to breed its own gilts. The herd followed practices of a 1-week batch production method and weaned piglets at the age of 28 days. The group allocation of weaned piglets was equal, according to body weight and sex, at flat-deck batteries with an

automatic climate-control system. The sows were vaccinated in a routine program against PRRSV, PCV2, swine influenza, porcine parvovirus, *Erysipelothrix rhusiopathiae*, *Clostridium perfringens* and *Escherichia coli*. Gilts were vaccinated against PRRSV twice before artificial insemination (180th and 210th days of age), and the sows underwent a 6th–60th vaccination scheme, using a commercial PRRSV MLV vaccine (Porcilis® PRRS, MSD Animal Health, Kenilworth, NJ, USA). Piglets were vaccinated against *Mycoplasma hyopneumoniae* and PCV2 at 21 days of age. The antiparasitic program of sows included injectable ivermectin once before farrowing, while boars were injected twice a year.

2.3. Health History and Pre-Trial Period

The trial farm had a previous PRRSV history, which included several severe outbreaks during the last ten years. PRRSV infection was verified by real-time polymerase chain reaction (qRT-PCR) examination performed in serum samples of sows/gilts as well as weaned, growing and finishing pigs). During past PRRSV outbreaks, clinical signs observed consisted of reproductive losses (increased abortion and returns to estrus rate, elevated number of mummified and stillborn piglets) accompanied with severe respiratory disease in the weaning stage associated with significant economic impact. Laboratory examinations in blood samples of unvaccinated suckling piglets for PRRSV showed a significant reduction of maternal antibodies against PRRSV at 14 days of age.

One month before the onset of the study, blood samples were taken from gilts, pregnant and lactating sows (12 total blood samples per group) and from suckling, weaned, growing and finishing pigs (8 samples at ages of 2, 4, 7, 10, 13, 17 and 21 weeks). Samples were screened for PRRSV genome by RT-PCR and the isolated viral strain was characterized via complete ORF5 Sanger sequencing. Additionally, the samples were examined for PCV2 by qRT-PCR.

Finally, nasal swab samples were tested by PCR for the detection of common respiratory pathogens (*Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Streptococcus suis*, *Pasteurella multocida* and *Haemophilus parasuis*).

2.4. Study Design

The trial included 104 healthy suckling piglets (2 weeks of age), derived from 9 litters. The piglets were divided into four groups (A–D, Table 1) of 13 piglets and two replicates of the experimentation were performed (2 replicates $\times$ 13 piglets $\times$ 4 groups/26 piglets per group).

Table 1. Results of qRT-PCR in blood serum samples of the sampled animals at ages of 4, 7 and 10 weeks.

Groups	Blood Sampling/Age		
	4 Weeks	7 Weeks	10 Weeks
	Number of PRRSV Positive Samples/Total Samples Average Ct Value (Min–Max)		
Group A (Porcilis PRRS ID)	0/6 N/A	0/6 N/A	6/6 33.3 (30.5–36.5)
Group B (Porcilis PRRS IM)	0/6 N/A	2/6 35.2 (32.3–38)	6/6 34.6 (29.5–39.7)
Group C (Diluvac ID)	0/6 N/A	5/6 34.7 (25.5–40.8)	6/6 29.4 (25.4–32.9)
Group D (Diluvac IM)	0/6 N/A	5/6 29.2 (24.2–32.2)	6/6 34.4 (25.6–39.0)

N/A: not applicable.

Diseased piglets and piglets with very poor condition (e.g., significantly low weight) or congenital abnormalities (e.g., hernias) were excluded from the trial. All animals included in the trial were distinguished by unique numbered ear tags and were housed and raised, mixed under the regular management program of the farm.

A commercial PRRSV MLV vaccine (Porcilis® PRRS, MSD Animal Health) was used in the current trial. The vaccine is approved for administration both via the IM or the ID way in the neck area, as stated in the Summary of Product Characteristics (SPC). Per vaccine dose of 2 mL (IM) or 0.2 mL (ID) of reconstituted lyophilized vaccine, the attenuated PRRSV strain DV is contained at titers 104.0–106.3 tissue culture infective dose 50%. Diluvac Forte®(MSD

Animal Health) is the adjuvant of the vaccine (containing 75 mg/mL dl- α tocopheryl acetate) that was administered to the piglets of the control groups.

Piglets from groups A and B were administered, at the age of 2 weeks, with one dose of Porcilis® PRRS, diluted in 0.2 mL (group A, ID) or 2 mL (group B, IM) of Diluvac Forte®. Piglets of groups C and D were administered with Diluvac Forte® only, using one dose of the recommended volume of adjuvant (0.2 mL for group C, or 2 mL for group D). An IDAL (IntraDermal Application of Liquids, MSD Animal Health) device was utilized to perform needle-free ID injection of experimental groups A and C. Pigs of IM administration groups (B and D) were injected with an automatic syringe (standard fixed volume 2 mL) and a new, sterile needle (size approx. 0.9 $\times$ 13 mm) was used for each group.

2.5. Sampling/Examinations

Blood samples were collected from each experimental group (three same ear-tagged piglets per group, for two replicates) at 4, 7 and 10 weeks of age, as shown in Figure 1. Blood serum samples were subjected to nucleic acid extraction with the use of the PureLink® Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Extracts were examined for the PRRSV genome, using an qRT-PCR assay [50]. Reactions were performed on a CFX96® Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). Cycle threshold (Ct) values were used as viral load estimates.

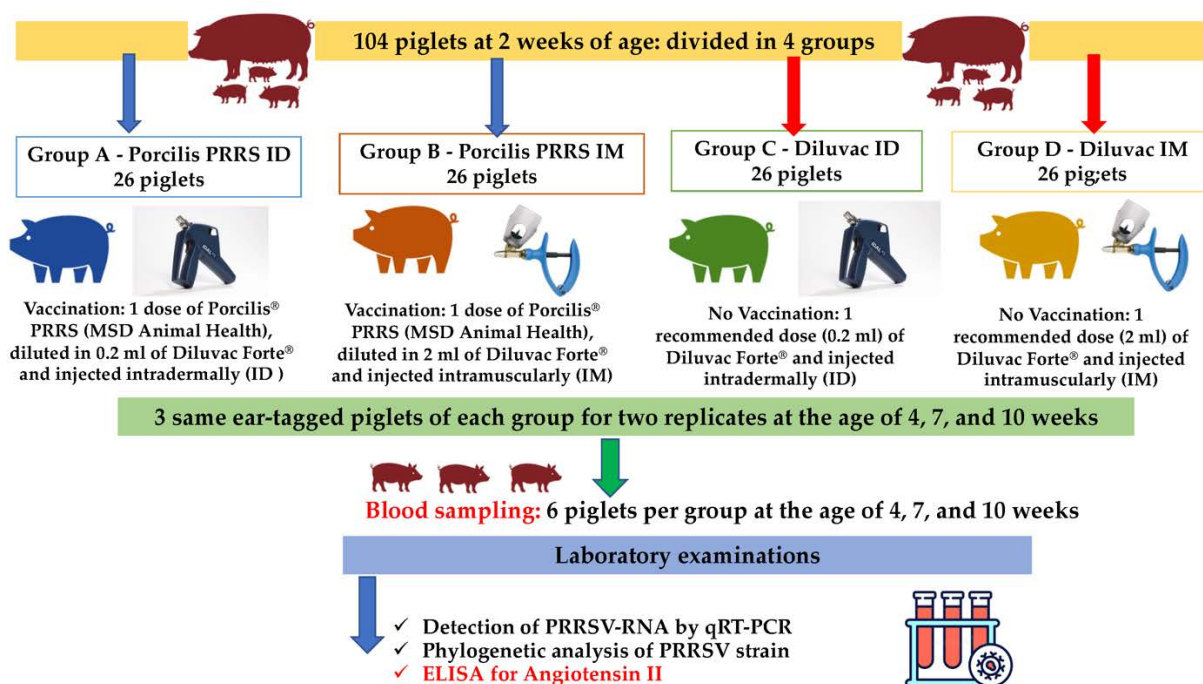


Figure 1. A flowchart of the trial design (experimental groups, vaccinations, sampling and laboratory examinations).

Serum samples were also tested by ELISA for the quantitative determination of Ang II levels (Pig angiotensin II, Ang II ELISA Kit; Catalogue number: CSB-E09370p, Cusabio, Wuhan, China). The method's characteristics included a quantitative sandwich enzyme immunoassay technique, a sensitivity of 1.56 pg/mL. The assay used has high sensitivity and excellent specificity for detection of pig Ang II. No significant cross-reactivity or interference between pig Ang II and analogues was noticed. The detection range was 6.25–400 pg/mL. The handling of blood samples was according to good laboratory practice.

In addition, nasal swab samples from the aforementioned six ear-tagged piglets per group were PCR tested for common respiratory pathogens (*Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Streptococcus suis*, *Pasteurella multocida* and *Haemophilus parasuis*).

2.6. Statistical Analysis

The distribution of continuous data was tested for normality using the Kolmogorov–Smirnov test and they are shown as mean $\pm$ standard error and/or median and range (non-normal distribution). Comparisons were done applying the Kruskal–Wallis test while the Mann–Whitney U test was utilized for post hoc/multiple comparisons. Significance was set at 0.05. Statistical analysis was performed in IBM (IBM Corp., Armonk, NY, USA).

Correlation between Ang II blood serum levels and PRRSV viral load as measured by qRT-PCR was evaluated by the non-parametric Spearman's correlation coefficient (ρ), using the commercial statistical software, MedCal 9.2 software (MedCalc Software, Mariakerke, Belgium). Significance was set at 0.05. The strength of the relationship was ranked as: $\rho \leq 0.35$ —weak correlations, 0.36 to 0.67—moderate correlations and 0.68 to 1.0—strong correlations [51].

3. Results

3.1. PCR Testing

PRRSV infection was verified before the onset of the trial by qRT-PCR in blood serum samples. Every sampled animal was qRT-PCR-positive for PRRSV. Analysis of the full-length ORF5 sequence of the wild-type PRRSV strain isolated in the trial farm at that time showed a 90.7% similarity (nucleotide sequence identity) with the DV strain (the data are available in Figure S1 of the Supplementary File). This strain is included in the commercial PRRSV MLV used in the current experiment. No positive samples for PCV2 were noticed.

The results of PCR testing for respiratory pathogens in nasal samples/during the pre-trial period were negative in all production stages that were sampled. Moreover, PCR screening for respiratory pathogens from experimental piglets revealed one positive unvaccinated pig at 7 weeks for *Streptococcus suis* and one positive unvaccinated pig at 10 weeks for *Streptococcus suis* and *Haemophilus parasuis*.

Findings of qRT-PCR in serum samples from pigs belonging to all groups at 4, 7 and 10 weeks of age are presented in Table 1. Specifically, pigs of all experimental groups were negative at the age of 4 weeks, and all pigs were positive at the age of 10 weeks, due to natural infection.

At the age of 7 weeks, all pigs of group A were negative, while 33.33% of pigs of group B and 83.33% of pigs of groups C and D were positive. However, the Ct value of qRT-PCR seems to be lower in group A compared to that of group B at the age of 10 weeks.

3.2. Angiotensin II Results

The results for Ang II levels are presented in Table 2. No significant differences were noticed in the same group over time. However, significant differences among groups were observed only at the age of 7 weeks (A vs. D, $p = 0.004$, B vs. D, $p = 0.037$, C vs. D, $p = 0.006$).

Table 2. Mean, standard error (SE), median and interquartile range (IQR) of angiotensin serum concentrations in all groups and time points.

Group	Time (Weeks)	Mean Angiotensin Concentration ($\times 10^2$ pg/mL)	SE	Median	IQR
Group A (Porcilis PRRS ID)	4	0.25	0.25	0.00	0.00–0.00
	7	0.26 ^a	0.21	0.06	0.00–0.14
	10	0.17	0.13	0.00	0.00–0.17
Group B (Porcilis PRRS IM)	4	2.97	1.86	0.90	0.09–3.63
	7	3.44 ^b	2.51	1.05	0.28–2.31
	10	3.20	1.80	0.65	0.20–6.63
Group C (Diluvac ID)	4	1.06	0.37	0.83	0.62–1.32
	7	0.56 ^a	0.42	0.08	0.03–0.45
	10	1.41	0.71	0.78	0.20–2.03
Group D (Diluvac IM))	4	4.13	2.30	1.14	0.17–7.52
	7	17.63 ^c	7.51	11.37	4.73–25.14
	10	2.93	2.05	0.19	0.01–3.64

^{a,b,c} Different superscript letters denote significant differences ($p < 0.05$).

3.3. *Correlation between PRRSV Viral Load and Ang II*

No significant correlation was noticed either in the total number of animals at both time points or separately in each time point.

4. Discussion

Vaccination is one of the most common preventive tools applied in the swine industry. Nowadays, various vaccinations are often applied until finishing age. Even if IM vaccination is the most applied method in pigs, negative consequences (e.g., pain and necrosis) have been reported [52]. The needle-free ID vaccination is a less invasive and less painful alternative to IM vaccination [52,53,54,55]. The skin, due to its high density of immunocompetent cells, could be an alternative target tissue for vaccination [56]. The pro-inflammatory activity of Ang II is also mediated by activation of dendritic cells, highly specialized antigen-presenting cells responsible for immune response and inflammation defense. However, no data are published regarding to the role of Ang II after IM or ID vaccination against PRRSV in swine. The ACE promotes the conversion of Ang I to vasoconstrictor Ang II and is present in many tissues [44]. Ang II plays a key role in inflammation, through oxidative stress and production of adhesion molecules and cytokines [32]. In our study, a significant difference of mean Ang II concentration between ID and IM in piglets at 7 weeks of age. The increased mean Ang II concentration in IM groups could be possible correlated with the increased inflammation. The mean Ang II concentration in IM groups was decreased later (at 10 weeks of age), maybe due to recovery of piglets from inflammation.

In our study, at 7 weeks, only group A (ID PRRSV vaccinated) was qRT-PCR negative and had lower Ang II levels in comparison to group B (IM PRRSV vaccinated). In particular, at the age of 7 weeks, all animals of group A were qRT-PCR negative, while 33.33% of animals belonging to group B and 83.33% belonging to groups C and D were qRT-PCR positive. This finding provides evidence that ID vaccination induces less tissue damage and maybe affect the

induction of sufficient protection. The diminished tissue damage is probably connected with lesser pain and distress. Behavioral alterations have been commonly utilized to assess pain and distress linked to injections in farm animals. A previous study, using the same commercial vaccine, demonstrated that ID-vaccinated suckling piglets exhibited more active behavior and suckling activity than piglets vaccinated through the IM route, 24 h after vaccination [57]. The needle-free ID vaccination method averts the acute phase response and muscle tissue damage related to IM injections [52]. Thus, ID vaccination offers important welfare benefits for the modern swine industry, as a less painful and less invasive alternative vaccine administration method when compared to IM injections [52].

Previous studies reported the regulatory role of PRRSV in induced apoptosis that are crucial to PRRSV pathogenesis [40,58,59,60]. Ang II, the key player of the renin-angiotensin system, has a crucial role in the inflammatory response, cell proliferation, migration and apoptosis [30,31,33]. In our previous study on ID and IM vaccination of piglets against PRRSV with the same vaccine, increased serum Fas levels were noticed in unvaccinated piglets, hinting apoptotic suppression when compared to vaccinated piglets. Moreover, higher sFAS levels were noticed the age of tested pigs increased, probably due to persistent PRRSV infection [61]. Ang II is a second important modulator of the FasL system in the lungs [41], while Fas/FasL is involved in PRRSV-induced apoptosis [42,43]. In our study, no significant differences in Ang II levels were noticed in the same group over time, while significant differences among groups were observed only at the age of 7 weeks. Due to Ang implication in the inflammation process, the absence of significant difference in Ang II levels in the same group could be attributed to the mild alteration of inflammation evidenced in all groups throughout the whole study period [32]. Moreover, no correlation between PRRSV viral load and Ang II was noticed in our study. Our results do not indubitably explain the role of Ang II in PRRSV-induced apoptosis. Previous studies, including comparison ID and IM MLV vaccination against PRRSV, reported that ID

vaccination effectively induces specific humoral and cellular immune responses at least the same or even superior to IM vaccination [10,11,62]. Our results could be useful for researchers studying the effect of ID vaccination in piglets. However, future studies, including increased number of experimental animals and samples, are required to explore further the potential role of Ang II in the PRRSV induced apoptosis and elicited an immune response from ID or IM vaccination.

In conclusion, the present study provides evidence that ID PRRSV vaccination induces diminished tissue damage, based on the lower levels of Ang II in the serum of ID vaccinated piglets.

Supplementary Materials

The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/vetsci9090496/s1>, Figure S1. Comparison of ORF 5 sequences between the DV vaccine strain and the wild-type virus circulating in the farm.

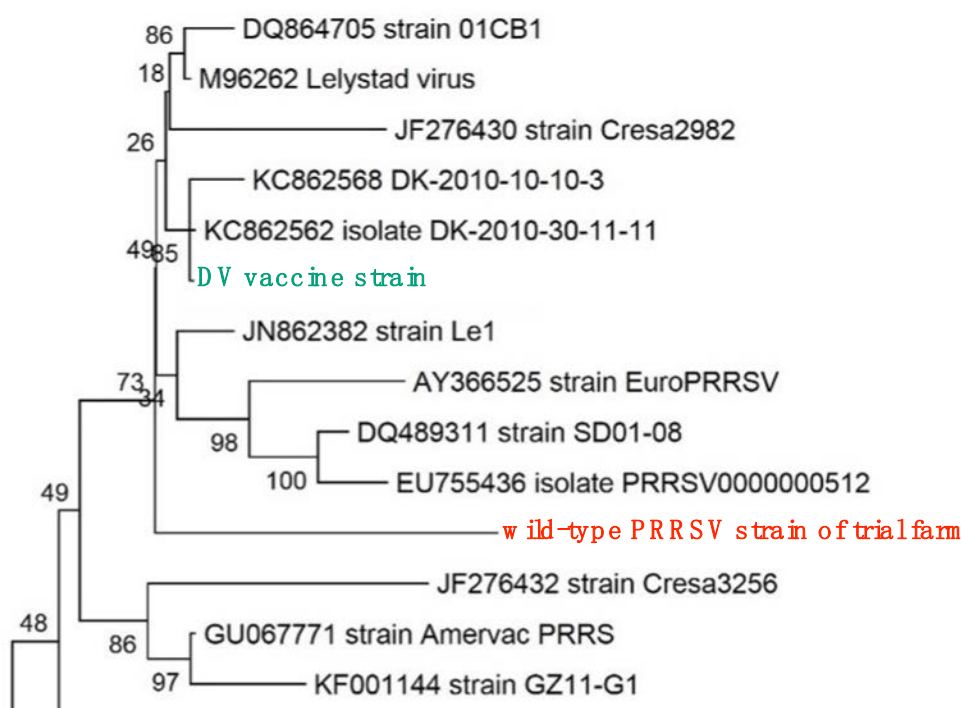


Figure S1. Comparison of ORF 5 sequences between the DV vaccine strain and the wild-type PRRSV strain of the trial farm: 90.7% sequence identity

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GENERAL CONCLUSIONS

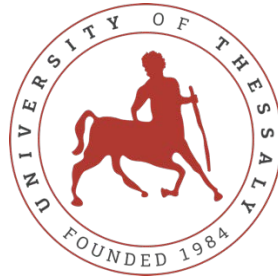
The studies carried out during this PhD thesis led to the following conclusions:

- The application of intradermal (ID) vaccination of suckling piglets with a commercial PRRSV-1 MLV vaccine (Porcilis® PRRS, MSD Animal Health at 2 weeks of age was safe and had beneficial effects benefits under field conditions, including improved body weight (BW) and lower Lung lesion score (LLS) and Pleurisy lesion score (PLS) index at slaughter age, as well as decreased mortality rate at growing/finishing stages.
- Significant indications that the vaccination of piglets with a commercial MLV vaccine against PRRSV via intradermal route had a positive effect on their protection against PRRSV viremia, equally to the use of the same vaccine utilizing intramuscular application.
- Based on the obtained ELISA and qRT-PCR results, indications were provided that the PRRSV-1 MLV vaccination of piglets by ID or IM route induce important seroconversion at 4 and 7 weeks of age (2-5 weeks post-vaccination) in comparison to non-vaccinated groups.
- Increased serum sFas levels in unvaccinated piglets were noticed, which could reveal apoptotic suppression compared to vaccinated piglets. No significant differences were noticed in serum sFas levels between ID and IM vaccination. Moreover, in the latter, vaccine-derived antibodies limit the infection and may attribute to the reduced Fas expression, suggesting a weak induction of lymphocyte-mediated cytotoxicity. Finally, higher sFAS levels were observed with aging, possibly due to persistent PRRSV infection.
- The analysis of our results demonstrated for the first time a significant positive and moderate correlation between PRRSV viral load and FAS level. This finding supports that PRRSV interferes with the induction of cellular innate immune response, pathways

of which are tied closely with that of apoptosis. This may explain why there is a delayed immune response in PRRSV-infected pigs and elucidation of the mechanisms of interference may provide clues into novel intervention strategies to combat PRRSV infection.

- A significant difference in mean Angiotensin II (Ang II) concentration between ID and IM in piglets at 7 weeks of age was noticed. The increased mean Ang II concentration in IM groups could be correlated with the increased inflammation. The mean Ang II concentration in IM groups was decreased later (at 10 weeks of age), maybe due to the recovery of piglets from inflammation.
- ID PRRSV vaccination induces diminished tissue damage, based on the lower levels of Ang II in the serum of ID-vaccinated piglets and may affect the induction of sufficient protection. The diminished tissue damage could be probably associated with lesser pain and distress.
- Moreover, no correlation between PRRSV viral load and Ang II was noticed in our study. Our results do not indubitably explain the role of Ang II in PRRSV-induced apoptosis.
- The experimental procedures and results of the current thesis provide beneficial information regarding the vaccination of piglets against PRRSV. Which is a matter that remains a big part of veterinary practitioners' and farmers daily challenge in the effort to efficiently manage the disease. However, future studies, including an increased number of experimental animals and samples, are required to explore further the part of sFAS levels after vaccination and the potential role of Ang II in the PRRSV-induced apoptosis and elicited immune response following ID or IM vaccination of piglets.

UNIVERSITY OF THESSALY



SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY MEDICINE

CLINIC OF MEDICINE

STUDY OF VACCINATION METHODS (INTRADERMAL, INTRAMUSCULAR) OF SUCKLING PIGLETS AGAINST PRRSV-1 ON THEIR HEALTH AND PERFORMANCE UNDER FIELD CONDITIONS

Doctoral Thesis by

Georgios G. Maragkakis

Summary

This thesis aimed to investigate vaccination methods (intramuscular-IM, intradermal-ID) against PRRSV for suckling piglets and their consequences on their health status and performance until they reached their slaughter age, under field conditions of natural infection. The novelty of our study is the first report of an ID vaccination on suckling piglets at the specific age of 14 days of age and, most importantly, under field conditions. Even though the ID vaccination of piglets against PRRSV is a common practice to the best of our knowledge, there are no published field studies investigating the effect of an ID vaccination on suckling piglets.

The first chapter concerns the results of the impact of the ID vaccination of piglets with a commercial PRRSV MLV vaccine (Porcilis® PRRS) in comparison to an IM vaccination on their health status and performance until their slaughter age, under field conditions, in a farm suffering from active PRRSV infection. The results of this study are important indications that the vaccination of piglets via the intradermal route had a positive effect on their protection against PRRSV viremia, equally of a vaccine with the intramuscular application. Based on the qRT-PCR results obtained by testing the serum samples, we noticed that the percentage of viremic animals in ID vaccinated piglets was lower at 10 weeks in comparison to IM vaccinated piglets, as well as the percentage of vaccinated groups in comparison to non-vaccinated groups at 7, 10, and 13 weeks. Based on the obtained ELISA and qRT-PCR results, indications were provided, that the PRRSV MLV vaccination of piglets by the ID or IM route induce an important seroconversion at 4 and 7 weeks of age (2–5 weeks post-vaccination) in comparison to non-vaccinated groups. In conclusion, this study demonstrated the safety of the ID vaccination of suckling piglets with a PRRSV MLV vaccine at 2 weeks of age under field conditions. No significant local and systemic reactions were noticed in vaccinated piglets until 14 days post-vaccination. Our study is the first report of an ID vaccination in suckling piglets at 14 days of age, indicating that both the ID and IM vaccination of piglets decreased the mortality rate, as well as the lung and pleurisy lesions score in vaccinated groups at the finishing stage. Moreover, the ID vaccination had a positive impact on the BW at the piglets' slaughter, while both the ID and IM vaccinations had a positive impact on the ADG.

The second chapter concerns the results from the investigation of the Fas (APO-1)-related apoptosis in piglets following intradermal (ID) and intramuscular (IM) vaccination with a PRRSV MLV in comparison with non-vaccinated piglets. Increased serum sFas levels in unvaccinated piglets were recorded, revealing apoptotic suppression compared to vaccinated piglets. In the latter, vaccine-derived antibodies limit the infection and may contribute to the

reduced Fas expression, suggesting a weak induction of lymphocyte-mediated cytotoxicity. Finally, higher sFas levels were observed with ageing, possibly due to persistent PRRSV infection. No significant differences were noticed in serum sFas levels between ID and IM vaccination. The analysis of our results demonstrated for the first time a significant positive and moderate correlation between PRRSV viral load and Fas levels. This finding supports that PRRSV interferes with the induction of cellular innate immune response, pathways of which are tied closely with that of apoptosis. This may explain why there is a delayed immune response in PRRSV-infected pigs and elucidation of the mechanisms of interference may provide clues into novel intervention strategies to combat PRRSV infection.

The last chapter concerns the results from the evaluation of the concentration of Ang II in the serum of piglets following immunization against PRRSV with an MLV vaccine, through the ID and IM route, compared to non-vaccinated piglets. No significant differences in Ang II levels were noticed in the same group over time, while significant differences among groups were observed only at the age of 7 weeks. Due to Ang implication in the inflammation process, the absence of significant difference in Ang II levels in the same group could be attributed to the mild alteration of inflammation evidenced in all groups throughout the whole study period. Moreover, no correlation between PRRSV viral load and Ang II was noticed in our study. Our results do not indubitably explain the role of Ang II in PRRSV-induced apoptosis. Our results could be useful for researchers studying the effect of ID vaccination of piglets. However, future studies, including an increased number of experimental animals and samples, are required to explore further the potential role of Ang II in the PRRSV-induced apoptosis and elicited an immune response from ID or IM vaccination. In conclusion, the present study provides evidence that ID PRRSV vaccination induces diminished tissue damage, based on the lower levels of Ang II in the serum of ID-vaccinated piglets.

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ



ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

ΠΑΘΟΛΟΓΙΚΗ ΚΛΙΝΙΚΗ

Μελέτη των μεθόδων εμβολιασμού γαλουχούμενων χοιριδίων (ενδοδερμικός, ενδομυϊκός), κατά του ιού του αναπαραγωγικού και αναπνευστικού σύνδρομού των χοίρων και των επιπτώσεων τους στην υγεία και στις αποδόσεις τους σε συνθήκες φυσικής λοίμωξης

Διδακτορική Διατριβή του

Γεωργίου Γ. Μαραγκάκη

Περίληψη

Η παρούσα διατριβή, είχε ως στόχο, τη διερεύνηση των μεθόδων εμβολιασμού (ενδομυϊκός-IM, ενδοδερμικός-ID) των θηλαζόντων χοιριδίων κατά του ιού του αναπαραγωγικού και αναπνευστικού σύνδρομού των χοίρων (PRRSV) και τις επιπτώσεις στην υγεία και στις αποδόσεις τους έως την ηλικία σφαγής τους, σε συνθήκες φυσικής λοίμωξης. Η καινοτομία της μελέτης είναι αποτελεί την πρώτη αναφορά ID εμβολιασμού των θηλαζόντων

χοιριδίων κατά του PRRSV στη συγκεκριμένη ηλικία των 14 ημερών και, κυρίως, σε συνθήκες φυσικής λοίμωξης. Παρόλο που ο ID εμβολιασμός των χοιριδίων κατά του PRRSV είναι μια κοινή πρακτική, δεν υπάρχουν δημοσιευμένες μελέτες πεδίου που να διερευνούν την επίδραση ενός ID εμβολιασμού στα θηλάζοντα χοιρίδια.

Στο πρώτο κεφάλαιο της διατριβής αναφέρονται τα αποτελέσματα των επιπτώσεων του ID εμβολιασμού χοιριδίων με ένα εμπορικό ελαττωμένης λοιμογόνου δύναμης (MLV) εμβόλιο κατά PRRSV (Porcilis® PRRS) σε σύγκριση με τον IM εμβολιασμό στην υγεία και τις αποδόσεις των εμβολιασμένων ζώων μέχρι την ηλικία σφαγής τους, συνθήκες φυσικής λοίμωξης. Τα αποτελέσματα αυτής της μελέτης αποτελούν σημαντικές ενδείξεις ότι ο εμβολιασμός των χοιριδίων μέσω της ενδοδερμικής οδού είχε θετική επίδραση στην προστασία τους έναντι της ιαιμίας από PRRSV, εξίσου με ένα εμβόλιο με ενδομυϊκή εφαρμογή. Με βάση τα αποτελέσματα qRT-PCR σε δείγματα ορού αίματος, παρατηρήθηκε ότι το ποσοστό των ιαιμικών ζώων στα ID-εμβολιασμένα χοιρίδια ήταν χαμηλότερο στις 10 εβδομάδες σε σύγκριση με τα IM-εμβολιασμένα χοιρίδια, καθώς και το ποσοστό των εμβολιασμένων ομάδων σε σύγκριση με τα μη -εμβολιασμένες ομάδες στις 7, 10 και 13 εβδομάδες. Με βάση τα αποτελέσματα ELISA και qRT-PCR, υπάρχουν ενδείξεις ότι ο εμβολιασμός των χοιριδίων κατά του PRRSV είτε με την ID οδό είτε με την IM οδό προκαλεί σημαντική ορομετατροπή στην ηλικία των 4 και 7 εβδομάδων (2-5 εβδομάδες μετά τον εμβολιασμό) σε σύγκριση με τις μη εμβολιασμένες ομάδες. Συμπερασματικά, αυτή η μελέτη έδειξε ότι ο ID εμβολιασμός των θηλαζόντων χοιριδίων κατά του PRRSV στην ηλικία 2 εβδομάδων είναι ασφαλής. Δεν παρατηρήθηκαν σημαντικές τοπικές και συστηματικές αντιδράσεις σε εμβολιασμένα χοιρίδια μέχρι τις 14 ημέρες μετά τον εμβολιασμό. Η μελέτη μας είναι η πρώτη αναφορά ID εμβολιασμού σε θηλάζοντα χοιρίδια ηλικίας 14 ημερών και υποδεικνύει ότι τόσο ο ID όσο και ο IM εμβολιασμός των χοιριδίων μειώνουν το ποσοστό θνησιμότητας, καθώς και τις αλλοιώσεις του πνεύμονα και της πλευρίτιδας στις εμβολιασμένες ομάδες. Επίσης, ο ID

εμβολιασμός είχε θετικό αντίκτυπο στο σωματικό βάρος (BW) κατά τη σφαγή, ενώ τόσο ο ID εμβολιασμός όσο και ο IM εμβολιασμός έχουν θετική επίδραση στην μέση ημερήσια αύξηση βάρους (ADG).

Στο δεύτερο κεφάλαιο περιλαμβάνονται αποτελέσματα από τη διερεύνηση της απόπτωσης που σχετίζεται με τον υποδοχέα Fas (γνωστός ως Apo-1 ή CD95) σε χοιρίδια μετά από ID και IM εμβολιασμό με εμπορικό MLV εμβόλιο κατά του PRRSV (Porcilis® PRRS) σε σύγκριση με μη εμβολιασμένα χοιρίδια. Καταγράφηκαν αυξημένα επίπεδα sFas στον ορό αίματος των μη εμβολιασμένων χοιριδίων, αποκαλύπτοντας αποπτωτική καταστολή σε σύγκριση με τα εμβολιασμένα χοιρίδια. Διαπιστώθηκε ότι πιθανώς τα αντισώματα που προέρχονται από το εμβόλιο περιορίζουν τη μόλυνση και μπορεί να συμβάλλουν στη μειωμένη έκφραση του Fas, υποδηλώνοντας ασθενή επαγωγή κυτταροτοξικότητας που προκαλείται από λεμφοκύτταρα. Τέλος, υψηλότερα επίπεδα sFas παρατηρήθηκαν στα χοιρίδια με την αύξηση της ηλικίας, πιθανώς λόγω επίμονης μόλυνσης από PRRSV. Δεν παρατηρήθηκαν σημαντικές διαφορές στα επίπεδα sFas στον ορό αίματος μεταξύ ID και IM εμβολιασμού. Η ανάλυση των αποτελεσμάτων μας έδειξε για πρώτη φορά μια σημαντική θετική και μέτρια συσχέτιση μεταξύ του ιικού φορτίου PRRSV και των επιπέδων Fas. Αυτό το εύρημα υποστηρίζει ότι το PRRSV παρεμβαίνει στην επαγωγή κυτταρικής έμφυτης ανοσοαπόκρισης, τα μονοπάτια της οποίας συνδέονται στενά με αυτή της απόπτωσης. Αυτό μπορεί να εξηγήσει γιατί υπάρχει καθυστερημένη ανοσολογική απόκριση σε χοίρους που έχουν μολυνθεί με PRRSV και η αποσαφήνιση των μηχανισμών παρεμβολής μπορεί να παρέχει ενδείξεις για νέες στρατηγικές στην αντιμετώπιση της λοίμωξης από PRRSV.

Το τελευταίο κεφάλαιο αναφέρεται στα αποτελέσματα από την αξιολόγηση της συγκέντρωσης της αγγειοτενσίνης II (Angiotensin II-Ang II) στον ορό αίματος των χοιριδίων μετά τον εμβολιασμό τους με εμπορικό MLV εμβόλιο κατά του PRRSV (Porcilis® PRRS), μέσω της ενδοερμικής (ID) και ενδομυϊκής (IM) οδού, σε σύγκριση με μη εμβολιασμένα χοιρίδια.

Δεν παρατηρήθηκαν σημαντικές διαφορές στα επίπεδα Ang II στην ίδια ομάδα με την πάροδο του χρόνου, ενώ σημαντικές διαφορές μεταξύ των ομάδων παρατηρήθηκαν μόνο στην ηλικία των 7 εβδομάδων. Λόγω της εμπλοκής της Ang στη διαδικασία της φλεγμονής, η απουσία σημαντικής διαφοράς στα επίπεδα Ang II στην ίδια ομάδα θα μπορούσε να αποδοθεί στην ήπια μεταβολή της φλεγμονής που παρατηρήθηκε σε όλες τις ομάδες κατά την συνολική περίοδο της μελέτης. Επιπλέον, δεν παρατηρήθηκε συσχέτιση μεταξύ του ιικού φορτίου του PRRSV και της Ang II στη μελέτη μας. Τα αποτελέσματά μας δεν εξηγούν αναμφίβολα τον ρόλο της Ang II στην απόπτωση που προκαλείται από τον PRRSV. Συμπερασματικά, η παρούσα μελέτη παρέχει σημαντικά στοιχεία ότι ο ID εμβολιασμός κατά τού PRRSV προκαλεί μειωμένη ιστική βλάβη, βάση των χαμηλότερων επιπέδων Ang II στον ορό αίματος των χοιριδίων που έχουν εμβολιαστεί μέσω της ενδοδερμικής οδού.

ANNEX I

CV author

Georgios Maragkakis

Georgios Maragkakis is a PhD candidate at the Clinic of Medicine, Faculty of Veterinary Medicine, University of Thessaly, Karditsa (Greece). After receiving his degree in Veterinary Medicine, he also completed a postgraduate internship in Farm Animal Medicine (specialization in porcine medicine) for one semester and a one-year postgraduate study program, specializing in porcine health management both at the Clinic of Medicine, Faculty of Veterinary Medicine, University of Thessaly, Karditsa (Greece). His author work includes 14 publications in scientific journals and 8 presentations at international conferences. He has also participated in several privately funded contract research programs, as a co researcher during his postgraduate studies at the Clinic of Medicine, Faculty of Veterinary Medicine, University of Thessaly, Karditsa (Greece). He has previously worked as a Key Account Manager of Swine and Poultry for Hipra Hellas S.A. and Key Account Manager Swine Greece & Cyprus for MSD Animal Health, both animal pharmaceutical companies. Currently he is working freelance and runs his privately owned veterinary practice.

ANNEX II

LIST OF ORIGINAL PUBLICATIONS

This thesis was based on the following original publications:

1. “Evaluation of Intradermal PRRSV MLV Vaccination of Suckling Piglets on Health and Performance Parameters under Field Conditions.”

Maragkakis G, Athanasiou LV, Chaintoutis SC, Psalla D, Kostoulas P, Meletis E, Papakonstantinou G, Maes D, Christodouloupoulos G, Papatsiros VG. *Animals*. 2023; 13(1):61. <https://doi.org/10.3390/ani13010061>

2. “Angiotensin II Blood Serum Levels in Piglets, after Intra-Dermal or Intramuscular Vaccination against PRRSV”

Maragkakis G, Athanasiou LV, Korou L-M, Chaintoutis SC, Dovas C, Perrea DN, Papakonstantinou G, Christodouloupoulos G, Maes D, Papatsiros VG. *Veterinary Sciences*. 2022; 9(9):496. <https://doi.org/10.3390/vetsci9090496>

3. “Investigation of Fas (APO-1)-Related Apoptosis in Piglets Intradermally or Intramuscularly Vaccinated with a Commercial PRRSV MLV”

Maragkakis G, Korou LM, Chaintoutis SC, Christodouloupoulos G, Dovas CI, Perrea D, Athanasiou LV, Konstantopoulos P, Maes D, Papatsiros VG. *Viral Immunol*. 2022 Mar;35(2):129-137. doi: 10.1089/vim.2021.0104.

4. “Exploring Serum Copeptin and Hematological Profile: A Comparative Analysis after Intradermal versus Intramuscular Porcine Reproductive and Respiratory Syndrome Virus Vaccination in Piglets”.

Maragkakis, G, Katsogiannou EG, Papakonstantinou GI, Korou L-M, Chaintoutis SC, Konstantopoulos P, Perrea DN, Christodouloupoulos G, Athanasiou LV; Papatsiros, VG. *Stresses* 2024, 4, 358-366. <https://doi.org/10.3390/stresses4020023>

List of other original publications:

1. Erythema Multiforme Associated with Respiratory Disease in a Commercial Breeding Pig Herd.

Papatsiros VG, Athanasiou LV, Psalla D, Petridou E, **Maragkakis GG**, Papatsas I, Arsenakis I, Maes D. *Viral Immunol.* 2015 Oct;28(8):464-71. doi: 10.1089/vim.2015.0063.

2. Cross-Sectional Serosurvey and Risk Factors Associated with the Presence of Toxoplasma gondii Antibodies in Pigs in Greece.

Papatsiros VG, Athanasiou LV, Stougiou D, Papadopoulos E, **Maragkakis GG**, Katsoulos PD, Lefkaditis M, Kantas D, Tzika ED, Tassis PD, Boutsini S. *Vector Borne Zoonotic Dis.* 2016 Jan;16(1):48-53. doi: 10.1089/vbz.2015.1845.

3. First-Time Detection of Porcine Reproductive and Respiratory Syndrome Virus and Porcine Circovirus 2 in an Albanian Farrow-to-Finish Herd.

Papatsiros VG, Chaintoutis SC, Psalla D, **Maragkakis GG**, Christodouloupoulos G, Dovas CI. *Viral Immunol.* 2018 Jun;31(5):397-403. doi: 10.1089/vim.2017.0166.

4. Notomelia in a piglet (Sus scrofa): Case report and review of the literature.

Pourlis A, Papatsiros VG, Athanasiou LV, **Maragkakis GG**, Tsioli V. *Anat Histol Embryol.* 2019 Jul;48(4):384-387. doi: 10.1111/ahe.12459.

5. First case of Serratia liquefaciens isolated from urinary tract infection in sows and associated clinicopathological and pathological findings.

Papatsiros VG, Athanasiou LV, Spanou VM, Stylianaki I, Papakonstantinou G, Letsios M, Villioti CS, Tsekouras N, **Maragkakis G**, Papaioannou N, Christodouloupoulos G. *Lett Appl Microbiol.* 2020 Apr;70(4):259-262. doi: 10.1111/lam.13267.

6. Effects of dietary fibre on metabolism and performance in sows.

Papatsiros VG, Katsarou MS, Drakoulis N, Maragkakis G, Tzika E, Maes D, Tassis PD, Lagiou M, Christodouloupoulos G. *Pol J Vet Sci.* 2021 Jun;24(2):271-279. doi: 10.24425/pjvs.2021.137662.

7. Occurrence and Associated Risk Factors of Porcine Reproductive and Respiratory Syndrome Virus and Porcine Circovirus Type 2 Infections in Greece.

Papatsiros VG, Papakonstantinou G, Meletis E, Tsekouras N, **Maragkakis G**, Bitchava D, Kostoulas P. *Viral Immunol.* 2022 Apr;35(3):200-211. doi: 10.1089/vim.2021.0124.

8. Histopathological Pulmonary Lesions in 1st-Day Newborn Piglets Derived from PRRSV-1 MLV Vaccinated Sows at the Last Stage of Gestation.

Papakonstantinou GI, Psalla D, Pourlis A, Stylianaki I, Athanasiou LV, Tzika E, Meletis E, Kostoulas P, **Maragkakis G**, Christodouloupoulos G, Papaioannou N, Papatsiros VG. *Life (Basel).* 2023 Jul 23;13(7):1609. doi: 10.3390/life13071609.

9. Seroprevalence of Swine Influenza A Virus (swIAV) Infections in Commercial Farrow-to-Finish Pig Farms in Greece.

Papatsiros VG, Papakonstantinou GI, Meletis E, Koutoulis K, Athanasakopoulou Z, **Maragkakis G**, Labronikou G, Terzidis I, Kostoulas P, Billinis C. *Vet Sci.* 2023 Sep 30;10(10):599. doi: 10.3390/vetsci10100599.

10. Effects of Injectable Administration of Dexamethasone Alone or in Combination with Vitamin E/Se in Newborn Low Birth Weight Piglets.

Papakonstantinou GI, Gougoulis DA, Voulgarakis N, **Maragkakis G**, Galamatis D, Athanasiou LV, Papatsiros VG. *Vet Sci.* 2023 Feb 9;10(2):135. doi: 10.3390/vetsci10020135.