Molecular characterization of the major envelope protein of porcine reproductive and respiratory syndrome virus (PRRSV) and evaluation of its use for a diagnostic assay, vaccine development, and the examination of quasispecies evolution

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Molecular characterization of the major envelope protein of porcine reproductive and respiratory syndrome virus (PRRSV) and evaluation of its use for a diagnostic assay, vaccine development, and the examination of quasispecies evolution

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is a viral disease that has devastated the global swine industry since the mid 1980s. Although modified live vaccines (MLVs) are typically used for the prevention of clinical disease, they are not always fully effective. Additionally, acute PRRS outbreaks, characterized by more severe clinical signs, have appeared in herds that were previously vaccinated. In this dissertation, we further analyzed the pathogenesis of PRRSV through genetic characterization, assay development, and quasispecies evaluation using the PRRSV ORF5 gene while also attempting to develop an improved PRRS vaccine.

To explore the possible mechanism for the emergence of acute PRRS, the open reading frame 5 (ORF5) gene encoding the major envelope protein (GP5) of acute PRRSV isolates was characterized. Sequence and phylogenetic analyses revealed that seven of the acute PRRS virus (PRRSV) isolates were related to other N. American PRRSV isolates while one isolate, 98-37120-2, was very closely related to and may have been derived from the MLV, RespPRRS. We also developed a heteroduplex mobility assay (HMA) for quickly identifying PRRSV field isolates with significant nucleotide sequence identities (≥98%) with the MLVs based on the amplification, denaturation, and reannealing of the ORF5 gene of the field isolates with those of MLV reference strains.

All of the field isolates that were highly related to RespPRRS (≤2% nucleotide sequence divergence) were identified by the HMA to form homoduplexes with the reference RespPRRS MLV.

We also developed a unique strategy for infecting pigs with PRRSV, known as *in vivo* transfection, by bypassing the traditional *in vitro* cell culture step required for *in vivo* studies. We demonstrated that inoculation of RNA transcripts of a PRRSV infectious cDNA clone directly into the lymph nodes and tonsils of pigs produces active PRRSV infection. Using this method, we also examined the quasispecies populations of PRRSV. Finally, we evaluated the ability of *Salmonella choleraesuis* to express the PRRSV GP5, and tested its immunogenicity in mice. Based on our data, there was no indication of *Salmonella* replication in the mice or any evidence of antibody production against *S. choleraesuis* or PRRSV GP5.

Dedication

I dedicate this dissertation to my parents, Audrey and William Key, and my grandmother, Audrey M. Farthing.

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General Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an emerging swine disease that was first recognized in the United States in 1987 (6). PRRS, often characterized by late-term abortions and stillbirths in sows and respiratory disease in nursery pigs, has resulted in extensive economic losses in the swine industry since its first appearance (13). By estimation, approximately half a billion dollars in annual losses to swine producers in the United States are due to PRRS outbreaks (20).

Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of PRRS, was first isolated in the Netherlands (24) using porcine alveolar macrophages and was designated as Lelystad virus (LV). Subsequently, the complete genome of LV was sequenced (15) and many other North American and European PRRSV isolates were characterized (2, 4, 5, 8, 9, 12). PRRSV is a small, enveloped, positive-stranded RNA virus consisting of eight overlapping open reading frames (ORFs) (15, 19). The virus is genetically, antigenically, and pathogenically heterogenic (13). Substantial sequence divergence exists between the European and North American genotypes of the virus, sharing only about 70% nucleotide sequence identity (1, 8, 9, 10, 12, 16, 17, 18). Among the North American isolates, the PRRSV genomic sequences also vary significantly (7, 10, 11, 18).

The heterogeneity of the virus has made it difficult to design effective vaccines based on a single PRRSV strain (13). Modified live-attenuated vaccines (MLVs) are currently used against PRRS and mainly provide protection against clinical disease (14,

22). These MLVs, such as Ingelvac PRRS MLV, have reduced the incidence and severity of PRRS outbreaks on many farms. A severe form of PRRS, designated acute or atypical PRRS, has been reported in the midwestern United States. Many of these acute outbreaks occurred in PRRSV MLV-vaccinated herds, suggesting that the commonly used MLVs are not fully effective. The origin of the acute isolates has not been determined.

Other concerns about the MLVs pertain to their safety. In Danish swine herds, Ingelvac PRRS MLV vaccine virus has been shown to be capable of reverting to a pathogenic phenotype (3, 21, 23). Additionally, Mengeling et al. (14) confirmed that numerous vaccine-like field isolates, which contained the same restriction enzyme site marker that is found in the Ingelvac PRRS MLV vaccine virus, were capable of causing disease much more severe than diseases induced by the MLV. The restriction enzyme site marker was not identified in any isolates collected prior to the introduction of the Ingelvac PRRS MLV vaccine except for its parent strain ATCC VR2332.

The emergence of new PRRS outbreaks in the presence of globally vaccinated herds along with the appearance of vaccine-related strains in herd outbreaks emphasizes the current need for additional research that examines both the diagnosis of the existing PRRSV isolates and the need for safer, more effective PRRSV vaccines. In this dissertation, we addressed these issues in an attempt to improve both the present and future state of the swine industry that has been so enormously impacted by this important pathogen.

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Chapter 1

Literature Review

Porcine Reproductive and Respiratory Syndrome Virus

History of porcine reproductive and respiratory syndrome (PRRS)

In the late 1980s, porcine reproductive and respiratory syndrome (PRRS) was recognized as an emerging disease that was causing severe outbreaks of reproductive failure and respiratory disease amongst U.S. swine herds. A few years later, a similar disease appeared in Europe and has since been identified throughout North America, South America, Europe, and Asia. In fact, PRRS is now endemic to most swine-producing nations. Although the disease is currently known as PRRS, it has also been referred to as swine infertility and respiratory syndrome (SIRS), porcine epidemic abortion and respiratory syndrome, mystery swine disease, and blue ear disease (9, 23, 52, 72, 111, 148).

Since its discovery, PRRS has resulted in extensive economic losses in the swine industry. A recent study calculated a loss of greater than 560 million dollars annually due to PRRS outbreaks in the U.S. (101). This estimate exceeds the combined annual losses from hog cholera and pseudorabies prior to their eradication. Despite exhaustive attempts to prevent and control the disease, its effects continue to greatly impact the global swine industry.

Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of PRRS, was first isolated by Wensvoort et al. (148). The European isolate was

named Lelystad virus (LV). Soon after the discovery of LV, PRRSV was also isolated and characterized in a number of continuous cell lines in North America (9, 23). Meulenberg et al. (91) first sequenced the complete genome of LV while partial sequences of other European (24) and North American isolates of PRRSV (74, 78) were also determined within a short period of time.

Since 1996, some PRRS outbreaks in the U.S. have been caused by a more virulent form of PRRSV resulting in a clinical syndrome that differs from that of a typical PRRSV infection. These PRRS outbreaks are characterized by an increased number of abortions in both early- and late-gestation and higher mortalities in herd outbreaks (12, 87). This severe type of PRRS is referred to as acute PRRS, atypical PRRS, or severe PRRS and often is not preventable by standard vaccination procedures (12).

Biology of PRRSV

General characteristics

PRRSV is a small, enveloped, positive-stranded RNA virus. Electron microscopic studies of PRRSV demonstrate a spherical particle approximately 58 nm in diameter with an inner nucleocapsid core (9, 26, 74, 149). The virus belongs to the order *Nidovirales*, along with the better known coronaviruses, and is a member of the family *Arteriviridae* (16, 128). Other arteriviruses include equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV).

Genomic organization

The viral genome of PRRSV is approximately 15 kb long and consists of eight overlapping open reading frames (ORFs) that are transcribed as a 3' nested set of subgenomic mRNAs. Seventy-five percent of the genome is designated as ORF1 consisting of ORF1a and ORF1b, which encode the nonstructural PRRSV proteins including those with replicase and polymerase activity. Recent studies of arteriviruses have revealed that a polyprotein is originally translated and then cleaved by proteases into several smaller nonstructural proteins (128). The remainder of the genome encodes the PRRSV structural proteins. ORFs 2–4 encode N-glycosylated virion-associated proteins designated as GP2, GP3, and GP4, respectively. ORFs 5–7 code for the major structural proteins including the major envelope (GP5), membrane (M) and nucleocapsid (N) proteins, respectively. The GP5 protein contains major neutralization epitopes and plays an important role in receptor recognition and viral infectivity (28, 109, 116) while the M protein forms a disulfide-linked heterodimer with GP5 and may also play a role in virus assembly, budding, and infectivity (76, 129, 156). The highly antigenic N protein is very abundant which explains why it is often used for diagnostic assays. Multimers of the N protein form the icosahedral structure of the virion.

The PRRSV major envelope protein, GP5, is a protein of particular interest in many PRRSV studies. The ORF5 gene encodes GP5 which is the most heterogeneic structural protein of the virus (53, 75, 80, 113, 135). GP5 consists of an N-terminal signal sequence that is 28-32 amino acids long, followed by an ectodomain region approximately 30 amino acids long with multiple N-glycosylation sites, a 70 aa-

hydrophobic transmembrane region, and a 66 aa-hydrophilic endodomain at the C-terminus. Various groups have identified multiple epitopes in GP5 (109, 112, 114, 116, 117, 147, 155, 160).

Life cycle

Porcine macrophages and monocytes are the cells most commonly infected by PRRSV during a natural infection although the virus has also been shown to infect testicular germ cells and pneumocytes (136, 137). PRRSV gains entry into macrophages by binding with heparan sulphate and sialoadhesin receptors followed by endocytosis (32, 33, 99, 144). Like other RNA viruses, PRRSV replicates in the cytoplasm where its antigens can be detected in the perinuclear region within a few hours post-infection (5, 161). Budding occurs at the membrane of the smooth endoplasmic reticulum or the Golgi apparatus and the virion particles are released by exocytosis (26).

In vitro, PRRSV is propagated in porcine alveolar macrophages (PAMs) and porcine blood monocytes as well as non-porcine cell lines from the African green monkey kidney cell line and its derivatives (MARC-145, CRL11171, and CL2621) (35, 55, 78, 81). Based on previous studies, European PRRSV strains have a greater affinity for the porcine cell lines (148, 150) while North American strains tend to thrive on PAMs as well as the non-porcine derivatives (9, 23, 55, 78, 81). PRRSV-infected PAMs and MARCs demonstrate cytopathic effect (CPE) characterized by clumping, rounding, and sometimes detachment of the cells within 2-4 days of infection (9, 55, 74, 148).

PRRSV in the animal

Pathogenesis

A PRRSV infection is initiated by viral invasion of local macrophages followed by a rapid spread to lymphoid organs and the lungs. Pulmonary alveolar macrophages, pulmonary intravascular macrophages, and macrophages in lymphoid tissues are the target cells for PRRSV replication (134). Peak viremia occurs between 7 to 14 days post-infection (dpi) which also coincides with the appearance of clinical signs. Generally, the viremia lasts for 28 days. Persistent infections do occur regularly and PRRSV may persist in the tonsils and lymph nodes following the clearance of viremia (126, 152).

The lesions that occur during a PRRSV infection often result from cellular injury induced by the virus. Studies indicate that PRRSV causes cell injury by inducing both indirect (35, 39, 43, 60, 85, 94, 127, 135, 138) and direct apoptosis (56), by generating proinflammatory cytokines (19, 139, 141, 145), and by activating polyclonal B cells in lymphoid tissues resulting in lymphoid hyperplasia (67). Typical gross lesions resulting from a PRRSV infection include consolidated lungs and markedly enlarged lymph nodes (125). Microscopically, PRRSV lesions include interstitial pneumonia, lymphocytic encephalitis, myocarditis, arteritis, and lymphoid hypertrophy and hyperplasia (49, 115, 122-124). Fetal lesions are less common and include edema of abdominal tissues, ascites, hydrothorax, and hydroabdomen (25, 62, 118). Occasionally, microscopic lesions are also found in the reproductive and renal systems (132).

Clinical features

PRRS outbreaks are most often characterized by reproductive failure in sows and respiratory disease in pigs of all ages, especially the young. During a PRRS epidemic, the first signs of infection are acute illness involving anorexia and lethargy, and sometimes pyrexia, dyspnea, and cyanosis of extremities, which rapidly spread throughout the herd over 7-10 days. Concurrently or soon after the acute illness, sows begin to experience increased reproductive failure for 1-4 months. A majority of the sows experiencing reproductive failure are those who were infected during their third trimester resulting in stillbirths, mummifications, and weak born piglets. Increased preweaning mortality also occurs during the months following a PRRS outbreak with rates as high as 60% (134). In addition to the classic acute signs, weanling and grower pigs may experience dyspnea, hyperpnea, cutaneous hyperemia, failure to thrive, and secondary bacterial infections. Once a population is endemically infected, sporadic outbreaks occur in susceptible populations such as newly introduced replacement gilts or sows and weaned pigs.

Despite the generalized description of the syndrome, the clinical picture of PRRS is extremely variable and is influenced by many viral, host, and environmental factors. The degree of both respiratory disease and reproductive disease may be affected by many of these factors. The virulence of the PRRSV isolate (49-51), the age or the stage of pregnancy at the time of infection, the presence of concurrent bacterial and viral infections (13, 38, 44, 140, 153), the farm management practices (45), and many other variables may all have an effect on the severity of a PRRSV infection.

Persistent infections often occur following an acute PRRSV infection. Experimentally, these infections have been shown to last for many months (3, 9, 23, 152, 154) and are characterized by lower viral loads predominantly in the lymphoid tissues including the tonsils and lymph nodes (9). During this period, the infected pigs appear to be recovered although the virus is still transmissible to susceptible pigs. This persistence of shedding is a major concern for the control and prevention of PRRS outbreaks (11). Although the mechanism of persistence is unknown, studies have documented that the virus can evade the immune response and persist in the lymphoid tissue for up to 157 days (152).

Immune response

Although the immune response to PRRSV is not fully understood, studies have shown that previously exposed pigs develop protective immunity against homologous PRRSV strains (64, 65) and sometimes heterologous PRRSV strains (65, 66, 88, 90). Clearly, both humoral and cell-mediated immunity are involved in the immune response against PRRSV. On the contrary, innate immunity, which would be the first defense against PRRSV as it enters the lungs, does not play a significant role. Lee et al. (68) demonstrated that the generation or suppression of interferon-alpha is quite variable based on the strain of PRRSV; however, there is an overall lack of interferon response following infection with PRRSV. In general, type I interferon is not strongly induced by PRRSV (1, 14, 22, 146).

Humoral immune response: Following PRRSV infection, a rapid antibody response is detected including an IgM response that occurs at 5-7 days post-infection (dpi) and an IgG response that is observed within 7-10 dpi (134). These initial antibodies are non-neutralizing (70, 162). Low levels of neutralizing antibodies appear approximately 3-4 weeks post-infection (1, 77, 162) and may play a significant role in clearance of viremia (59, 98, 108, 163). While the GP4 protein was the first identified neutralizing region of PRRSV (92), PRRSV neutralizing antibodies are primarily generated against the PRRSV major envelope protein, GP5 (47, 109, 112, 114, 147, 160). Various studies have demonstrated the presence of both neutralizing and nonneutralizing epitopes on GP5 (109, 112, 114, 116, 117, 147, 155, 159). When inoculated into mice and pigs, the GP5 protein has generated anti-GP5 neutralizing monoclonal antibodies while subsequent studies confirmed that these specific anti-GP5 monoclonal antibodies protected against lesions and clinical disease in PRRSV-challenged pigs (112, 113, 157). Additionally, the key role of antibodies in protection from PRRSV has been demonstrated in animal studies showing that passive immunity was conferred by delivering PRRSV antibodies to pigs that were later protected during challenge with a homologous PRRSV strain (65, 70, 108).

Antibody dependent enhancement (ADE) has been demonstrated in pigs with sub-neutralizing levels of PRRSV antibodies. In the case of PRRSV, these antibodies facilitate the entry of the virus into macrophages and can increase the level and duration of viremia (163). Antibodies against the GP5 and N proteins have been identified as ADE facilitators (15).

Cell-mediated immunity: A T-cell mediated immune response, generated by CD4+ and CD8+ T-cell proliferation, is elicited in response to PRRSV infection beginning at 4 weeks post-infection and lasting 5-10 weeks (6, 71). Bautista et al. (7) demonstrated that the T-cell response mainly targets the PRRSV M and GP5 proteins. Interferon-gamma, a T-helper 1 cytokine, has been shown to block PRRSV replication (8), however, Meier et al. (77) identified a gradual and weak interferon-gamma response following PRRSV infection. A delayed-type hypersensitivity (DTH) response has also been observed in response to PRRSV infection (6).

PRRSV in the population

Transmission

Both horizontal and vertical transmissions play a role in the spread of PRRSV. Horizontal transmission commonly occurs by direct contact between an infected pig and a naive pig involving an exchange of nasal secretions, saliva, urine, or semen. Studies examining fecal shedding of PRRSV are inconsistent. The viral titer required to infect a pig parenterally has been reported to be as small as 20 viral particles (164) while the titers required to infect a pig intranasally or orally are significantly larger. Therefore, parental inoculation routes, including bites or abrasions from other pigs, husbandry and medical procedures, or any other circumstances in which the skin is damaged, in addition to mucosal contact, are considered the predominant routes of horizontal transmission. Artificial insemination also promotes the spread of PRRSV (48, 158). PRRSV shedding

has been detectable in the semen of boars up to 92 days post-inoculation (21). Fomites are another source of infection particularly during the winter months when the enveloped virus can survive in more favorable environmental conditions (30, 31, 110). The role of aerosol transmission is still highly debated while the transfer of PRRSV by insects and waterfowl has also been discussed and is not fully understood. Vertical transmission typically occurs when a sow is infected during gestation, usually within the third trimester, which is the optimal time for a PRRSV transplacental infection (20, 61, 84, 120).

Heterogeneity

PRRSV is genetically, antigenically, and pathogenically heterogeneous (83). Based on distinct genetic variation, PRRSV isolates are divided into two genotypes, European and North American (2, 79, 91, 96, 97, 100). Substantial sequence divergence exists between the European and North American genotypes of the virus, sharing only about 70% nucleotide sequence identity (54, 79). A significant yet smaller divergence also exists amongst North American PRRSV isolates (2, 4, 42, 46, 53, 54, 80, 97, 100, 114, 151). In fact, genetically divergent PRRSV isolates have even been found within the same herd population (29, 46). Recently, European-like isolates have also been identified in North America. These isolates share approximately 95% nucleotide sequence identity with the prototype European strain and contain a small deletion in ORF1b (36, 121).

Antigenic and pathogenic variations are consequences of PRRSV genetic

heterogeneity. Antigenically, the North American and European PRRSV genotypes have been found to be quite distinct based on serological surveys (17, 34, 41, 69), indirect fluorescence assays (27, 73), and western blot analysis (57). The pathogenic difference of PRRSV isolates is demonstrated by the variation in severity of clinical respiratory disease (49-51) and reproductive failure (86) amongst infected swine. Both high- and low- virulence isolates are found within the United States (49, 80). The recent acute PRRS outbreaks (12, 87) and apathogenic PRRSV strains (105, 143) are further examples of the extreme variations amongst the isolates.

Control and Eradication

Modified live-attenuated vaccines (MLVs) are currently used for the prevention of PRRS outbreaks mainly by providing protection against clinical disease (88, 107). These modified live-attenuated vaccines, such as Ingelvac RespPRRS MLV, have reduced the incidence and severity of PRRS outbreaks on many farms. Despite their success rate, the MLVs are not completely safe or effective. For example, acute PRRS outbreaks have occurred in already-vaccinated herds and the Ingelvac RespPRRS MLV vaccine virus has been shown to be capable of reverting to a pathogenic phenotype (12, 106, 133). Serum inoculation and killed vaccines have also been used in attempts to control the disease but have proven less effective than the MLVs (37, 102, 104). Additionally, the heterogeneity of PRRSV has made it difficult to design effective vaccines based on a single PRRSV strain (83). Attempts have also been made to eradicate PRRS from populations. These methods including whole herd depopulation,

test and removal, and herd closure were found to be effective yet costly (18). The evidence of vaccine failure and the inability to sensibly eradicate PRRS outbreaks demonstrates the obvious need for more improved vaccines, better diagnostics, and more stringent biosecurity regulations. The recent development of PRRSV infectious cDNA clones (58, 93, 103, 142) provides a tool for the manipulation of the viral genome, thereby increasing the likelihood of developing genetically-engineered vaccines and better understanding the mechanisms of PRRSV replication and pathogenesis in the future.

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Chapter 2

Genetic variation and phylogenetic analyses of the ORF5 gene of acute porcine reproductive and respiratory syndrome virus isolates

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ABSTRACT

Swine herds in the U.S. have experienced recent outbreaks of a severe form of porcine reproductive and respiratory syndrome (designated acute or atypical PRRS) characterized by abortion and high mortality in pregnant sows. Most of the affected herds had been vaccinated with modified-live vaccines (MLVs) against PRRS. To explore the possible mechanism of the emergence of acute PRRS, the ORF5 gene encoding the major envelope protein (GP5) of acute PRRSV isolates was characterized. The complete ORF5 gene of eight acute PRRSV isolates from herds experiencing acute PRRS outbreaks in Iowa and North Carolina was amplified and sequenced. Sequence analyses revealed that these acute PRRSV isolates shared 88-95% nucleotide and 88-96% amino acid sequence identities to each other, 87-97% nucleotide and 84-96% amino acid sequence identities with other North American PRRSV isolates and the MLVs. Most of the amino acid substitutions locate in the putative signal sequence and two short hypervariable regions at the amino terminus. The ORF5 gene sequence of the acute PRRSV isolate 98-37120-2 from a non-vaccinated swine herd in Iowa is very closely related to that of the RespPRRS MLV,

with 97% nucleotide and 96% amino acid sequence identities. Phylogenetic analysis revealed that all eight acute PRRSV isolates are clustered within the North American genotype. Several minor branches that are not associated with geographic origins were also identified within the North American genotype. One acute PRRSV isolate (98-37120-2) is clustered with the RespPRRS MLV and several Danish isolates that were confirmed to be derived from the RespPRRS MLV. The ORF5 gene sequences of other seven acute isolates are more related to those of several earlier PRRSV isolates and the PrimePac MLV than to that of the RespPRRS MLV. Our results showed that the acute PRRSV isolates analyzed in this study differed from each other in ORF5 genes, although they all clustered within the North American genotype. The data from this study do not fully support the hypothesis that the emergence of acute PRRS is due to reversion of MLVs to a pathogenic phenotype, as only one of the eight acute isolates was shown to be very closely related to the RespPRRS MLV.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), characterized by severe reproductive failure in sows and respiratory diseases in young pigs, was first recognized in the U.S. in 1987 (Hill, 1990). Since its first appearance, PRRS has been causing immense economic losses in the swine industry (Polson et al., 1992). The causative agent of PRRS, porcine reproductive and respiratory syndrome virus (PRRSV), was first isolated by Wensvoort et al (1991) in the Netherlands by using porcine alveolar macrophages and was designated as Lelystad virus (LV). In the U.S., PRRSV was

isolated and characterized in a number of continuous cell lines (Benfield et al., 1992; Collins et al., 1992; Meng et al., 1994, 1996a). Meulenberg et al. (1993) first sequenced the complete genome of LV. Subsequently, partial sequences of another European isolate (Conzelmann et al., 1993) and two North American isolates of PRRSV (Mardassi et al., 1994; Meng et al., 1994) were reported. The genome of PRRSV is a positive strand RNA molecule of about 15 kb that encodes eight overlapping open reading frames (ORFs) (Meulenberg et al., 1993; Nelsen et al., 1999). The ORF1 is believed to encode the nonstructural proteins. It has been shown that the ORFs 2, 3 and 4 encode virion-associated proteins designated as GP2, GP3 and GP4, respectively (Meulenberg et al., 1995; Van Nieuwstadt et al., 1996). ORFs 5, 6 and 7 encode the major envelope (GP5), membrane (M) and nucleocapsid (N) proteins, respectively (Mardassi et al., 1996; Meulenberg et al., 1995). It has been shown that monoclonal antibodies directed against GP4 and GP5 proteins are neutralizing (Pirzadeh and Dea, 1997). PRRSV, along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV), is now classified within a single genus *Arterivirus*, in the family *Arteriviridae* in the order *Nidovirales* (Cavanagh, 1997).

PRRS has now been recognized worldwide and is considered to be a global disease with important economic impact. Although PRRSV isolates identified from around the world cause similar diseases in pigs, increasing data indicates that PRRSV is antigenically, genetically and pathologically very heterogenic (Andreyev et al., 1997; Halbur et al., 1995, 1996a, 1996b; Kapur et al., 1996; Meng et al., 1994, 1995a, 1995b, 1996a, 1996b, 2000; Murtaugh et al., 1995). Despite the availability of several PRRSV vaccines, the disease remains difficult to control. Recently, a more virulent form of

PRRSV has been causing high abortion and mortality rates in vaccinated swine populations in the U.S. (Bell, 1998; Botner et al., 1997; Bush et al., 1999; Halbur and Bush, 1997; Lager et al., 1998; Mengeling et al., 1998). These severe outbreaks of PRRS have been referred to as acute PRRS, atypical PRRS, hot PRRS, abortion storm, or sow abortion and mortality syndrome. The term "acute PRRS" is used throughout this manuscript. Zimmerman et al. (1997) described the criteria for the diagnosis of acute PRRS which include acute onset, clinical signs occurring over a 2- to 4-week period, high mortality (>5%) in sows and boars, and a high rate of abortions (>10%). Many of the herds affected by acute PRRS were on a PRRS vaccination program with the available modified-live vaccines (MLVs), suggesting that the vaccine-induced antibodies failed to neutralize the acute PRRS virus.

There are no published data on the genetic characterization of acute PRRSV isolates, and the mechanism for the emergence of acute PRRS in vaccinated pigs is still not known. In pigs vaccinated with MLVs, both cell-mediated immunity and humoral immune responses against the GP5 and other structural proteins of PRRSV play important roles in protection against PRRS. The GP5 protein is exposed outside the virion (Meulenberg et al., 1995) and thus is constantly under positive or negative selection forces. It has been shown that the ORF5 gene sequence of PRRSV is very polymorphic (Andreyev et al., 1997; Kapur et al., 1996). Therefore, genetic comparison of the ORF5 gene sequences of the acute PRRSV isolates to those of MLVs and other PRRSV isolates identified prior to acute PRRS outbreaks may provide insight into the genetic evolution and origin of the acute PRRSV isolates.

MATERIALS AND METHODS

Acute PRRSV isolates

The eight acute PRRSV isolates used in this study were obtained from the National Veterinary Services Laboratories in Ames, Iowa. The diagnosis and isolation of the acute PRRS isolates have been previously reported (Bush et al., 1999). The acute PRRSV isolates were from pigs that had experienced acute PRRS outbreaks in Iowa and North Carolina (Table 1). Each virus isolate was from a separate herd with acute PRRS. Seven of the eight acute PRRSV isolates were from herds that had been vaccinated with the available MLVs: four herds with RespPRRS MLV alone, three herds with both RespPRRS and PrimePac MLVs. One of the acute PRRSV isolates was isolated from a non-vaccinated herd in Iowa (Table 1).

Degenerate primers to amplify ORF5 gene of acute PRRSV isolates

The ORF5 gene is very heterogenic among different isolates of PRRSV. Therefore, a set of degenerate PCR primers was designed in order to amplify the ORF5 gene of the acute PRRSV isolates. The design of primers was based on a multiple sequence alignment of the ORFs 2 to 7 genes of 26 available PRRSV isolates. The sense primer (ORF5U, 5'-GGTGGGCAACKGTTTTAGCCTGTC-3') was located 37 bp upstream of the start codon of ORF5, and the antisense primer (ORF5L, 5'-GGTAATAGARAAYGCCAAAAGCACC-3') was located 34 bp downstream of the ORF5 stop codon. The primers were synthesized and purified commercially (GIBCO-BRL, Gaithersburg, MD). The expected PCR product of 723 bp contains the complete ORF5 gene.

RNA extraction and reverse transcriptase PCR (RT-PCR)

Total RNA was extracted with TriZol Reagents (GIBCO-BRL) from 100 µl of each of the eight acute PRRSV isolates propagated once in ATCC CRL11171 cells (Meng et al., 1996a). The total RNA was resuspended in 11.5 µl of DNase-, RNase- and Proteinase-free water (Eppendorf, Inc). Reverse transcription was performed at 42°C for 60 min in the presence of a reaction mixture consisting of 11.5 µl of the total RNA, 1µl of Superscript II reverse transcriptase (GIBCO-BRL), 1µl of 10 µM antisense primer, 0.5µl of RNase inhibitor (GIBCO-BRL), 1µl of 0.1 M dithiothreitol, 4 µl of 5X RT buffer, and 1µl of 10 mM dNTPs. The resulting cDNA was amplified by PCR with ORF5 sense and antisense degenerate primers and Ampli*Taq* Gold DNA polymerase (Perkin-Elmer). The PCR reaction was carried out for 5 cycles of denaturation at 94^0 C for 1 min, annealing at 45^0 C for 30 sec, extension at 72^0 C for 1 min 20 sec, and 34 cycles of denaturation at 94^0 C for 1 min, annealing at 52^0 C for 40 sec, extension at 72^0 C for 1 min 10 sec, and followed by a final incubation at 72^0 C for 7 minutes. The amplified PCR products were examined by gel electrophoresis.

Nucleotide sequencing

The expected PCR products amplified from each of the eight acute PRRSV isolates were excised from the agarose gel. The PCR products were subsequently purified using the glassmilk procedure with a GENECLEAN kit (Bio 101, Inc.). After measuring the DNA concentration, the PCR products were sequenced at the Virginia Tech DNA Sequencing Facility with an Automated DNA Sequencer (Applied Biosystem, Inc).

Sequences of the PCR products were determined for both DNA strands. The ORF5 sequences of the 8 acute PRRSV isolates reported in this paper have been deposited with the GenBank database under the accession numbers AF339493, AF339494, AF339495, AF339496, AF339497, AF339498, AF339499, and AF339500.

Sequence and phylogenetic analyses

The complete sequences of the ORF5 gene of the eight acute PRRSV isolates were analyzed with the MacVector (Oxford Molecular, Inc.) and DNAstar (DNASTAR, Inc) computer programs. The ORF5 gene sequences of other known PRRSV isolates used in this study were retrieved from the GenBank (Table 1). The percentages of sequence identity among different PRRSV isolates were determined with the MacVector program. Phylogenetic analysis was conducted with the aid of the PAUP program (David L. Swofford, Smithsonian Institute, Washington, DC, distributed by Sinauer Associates, Inc., Sunderland, MA). Heuristic search with 1,000 replicates and midpoint rooting options was used to produce a consensus tree.

RESULTS

Sequence and phylogenetic analyses of the ORF5 genes of acute PRRSV isolates and those isolated prior to the acute PRRS outbreaks

The complete ORF5 gene of eight acute PRRSV isolates was amplified by RT-PCR with a set of degenerate primers. Sequence analyses revealed that the ORF5 genes of the 8 acute PRRSV isolates characterized in this study all have the same size of 603 bp

as those of most other PRRSV isolates. Among the eight acute PRRSV isolates studied, they shared 88-95% nucleotide and 88-96% amino acid sequence identities in the ORF5 gene (Table 2). Most of the amino acid substitutions locate in two short hypervariable regions at the amino terminal region (amino acid positions 32-39 and 57-61) (Fig. 1). The ORF5 gene sequences of the 8 acute PRRSV isolates shared 87-97% nucleotide and 84-96% amino acid sequence identities with those of other PRRSV isolates within the North American genotype. The ORF5 gene sequences of the 8 acute PRRSV isolates shared only 61-63% nucleotide and 54-57% amino acid sequence identities with those of most European PRRSV isolates, with the exception of several Danish isolates that were confirmed to be North American RespPRRS MLV derivatives (Madsen et al., 1998; Fig. 2).

To gain a better understanding of the genetic relationship and evolution of acute PRRSV, phylogenetic analysis was performed based on the sequences of the ORF5 gene of the 8 acute PRRSV isolates from this study and 82 other known PRRSV isolates worldwide (Table 1). Phylogenetic analysis confirms the existence of two distinct genotypes of PRRSV: the North American genotype and the European genotype (Fig. 2). Interestingly, several recent Danish isolates, two Taiwanese isolates (MD001 and FI), two Chinese isolates (S1 and CH1a), a Japanese isolate (Kitasato 931), and an isolate from Guatemala (249010) are all clustered within the North American genotype. The North American genotype of the Danish isolates has been confirmed to be derived from the RespPRRS vaccine that was used in Danish swine herds (Madsen et al., 1998). The origins of the Asian isolates with a North American genotype are not known. Within the major North American genotype, several minor branches (or minor genotypes) were also

observed (Fig. 2). However, these minor branches do not appear to be associated with geographic origins of PRRSV isolates. All of the 8 acute PRRSV isolates analyzed in this study belong to the North American genotype. Seven of the eight acute isolates from Iowa and North Carolina are clustered with several recent Illinois isolates, a few Asian isolates and are more related to the PrimePac vaccine than to the RespPRRS vaccine (Fig. 2). The other acute isolate (98-37120-2) ORF5 gene sequence is most closely related to that of RespPRRS vaccine and its parent virus VR2332 as well as with several Danish PRRSV isolates that were confirmed to have been derived from the RespPRRS vaccine (Madsen et al., 1998).

Comparison of the ORF5 gene sequence of acute PRRSV isolates with that of MLVs

The ORF5 gene sequence of the acute PRRSV isolate 98-37120-2 is more closely related to that of the RespPRRS MLV and its parent virus VR2332 (97% nucleotide and 96% amino acid sequence identities, respectively) than to that of most other PRRSV isolates (Fig. 2, Table 2). There are a total of 8 amino acid substitutions between the acute PRRSV isolate 98-37120-2 and the RespPRRS MLV in the entire GP5 amino acid sequence. Seven of the 8 substitutions locate in the heterogeneic signal sequence and the hypervariable regions (Fig. 1). The other substitution occurs at amino acid position 151, where a glycine residue in the RespPRRS MLV is replaced by an arginine residue in acute PRRSV isolate 98-37120-2. This glycine-to-arginine change at amino acid position 151 has been used as a marker to study the change of the restriction fragment length polymorphism (RFLP) patterns as the vaccine virus spreads among a swine population

(Wesley et al., 1999). Interestingly, the virulent parent strain of RespPRRS MLV (isolate VR2332) also has an arginine residue at position 151.

The ORF5 gene sequences of the other 7 acute PRRSV isolate are more related to that of the PrimePac MLV than to that of RespPRRS MLV (Fig. 2). However, the sequence identities of the 7 acute isolates with the PrimePac MLV do not exceed those observed between the acute isolates and other PRRSV isolates. The ORF5 gene sequences of seven other acute PRRSV isolates share 90-92% nucleotide and 89-94% amino acid sequence identities with that of the PrimePac MLV (Table 2). The amino acid substitutions of the GP5 protein between the 7 acute PRRSV isolates and PrimePac MLV are mostly in the heterogenic signal sequence and the two hypervariable regions. However, amino acid substitutions in other regions of the GP5 protein are also observed (Fig. 1).

DISCUSSION

The emergence and reemergence of viral infectious diseases is often influenced by the genetics of the viruses (Domingo and Holland, 1992; Duarte et al., 1994). Extensive genetic variation has been observed among PRRSV isolates, and the observed genetic heterogeneity of PRRSV could lead to the selection of more virulent viruses and to the emergence or reemergence of new forms of PRRS. Quasispecies evolution of PRRSV has been reported in PRRSV infected pigs (Rowland et al., 1999), and this may significantly change the genomic sequence of MLVs over time as the vaccine virus spreads among swine herds. Ultimately, these genetic changes could cause the reversion of MLVs to virulent phenotypes. RNA recombination has also been reported in PRRSV

as a mechanism of generating heterogeneity (Yuan et al., 1999). Thus it is possible that virulent strains of PRRSV could be generated through RNA recombination between MLVs used in the vaccination programs and enzootic field strains of PRRSV (Meng, 2000).

The mechanism of the emergence of acute PRRSV is not known. The intriguing fact is that most of the herds affected by the acute PRRS had been vaccinated with the current MLVs (Bell, 1998; Bush et al., 1999; Halbur and Bush, 1997; Mengeling et al., 1998; Lager et al., 1998), suggesting that the acute PRRSV is antigenically distinct from the MLVs. In this study, we determined the complete sequences of the ORF5 gene of 8 acute PRRSV isolates. Each of these 8 acute PRRSV isolates was isolated from a separate herd with acute PRRS outbreaks in Iowa and North Carolina. Seven of the 8 acute PRRSV isolates were isolated from herds that had been vaccinated with either RespPRRS alone or with both RespPRRS and PrimePac MLVs. The RespPRRS/Repro^{TM} (Boehringer Ingelheim, Inc.), an MLV, is recommended for use in 3-18 week old pigs and in non-pregnant females (Dee and Joo, 1997). The PrimePac PRRS vaccine (Schering Plough Animal Health Corporation) (Hesse et al., 1997) is also an MLV which has been shown to reduce the severity and duration of disease following challenge. Sequence analyses revealed that the 7 acute PRRSV isolates identified from vaccinated herds are clustered with numerous recent Illinois isolates, two Asian isolates (Taiwan MD001 and China CH1a), a Guatemala isolate, and numerous other North American isolates. The ORF5 gene sequence of these 7 acute PRRSV isolates shared 90-92% nucleotide sequence identities with that of the PrimePac MLV, and 88-90% nucleotide sequence identities with that of the RespPRRS MLV. Therefore, it appears

that these 7 acute PRRSV isolates identified from vaccinated herds are not direct derivatives of the MLVs that were used in swine herds in the U.S. It has been shown that antibodies directed against GP5 protein are neutralizing. Therefore, the observed genetic differences in GP5 gene sequence between acute PRRSV isolates and MLVs may render these acute PRRSV isolates the ability to escape the immune response induced by the RespPRRS and PrimePac MLVs, and thereby causing acute PRRS outbreaks in vaccinated herds.

One of the acute PRRSV isolates examined in this study, isolate 98-37120-2, was isolated from a non-vaccinated swine herd in Iowa (Table 1). Interestingly, the ORF5 gene sequence of this particular acute PRRSV is very closely related to that of the RespPRRS MLV, with 97% nucleotide and 96% amino acid sequence identities. There are only eight amino acid differences in ORF5 between this acute PRRSV isolate and the RespPRRS MLV, and 7 of the 8 amino acid substitutions locate in the heterogenic signal sequence and the hypervariable regions at the amino terminus. The other amino acid substitution locates at position 151 where a glycine in RespPRRS MLV is replaced by an arginine in both acute PRRSV isolate 98-37120-2 and the virulent parent strain of RespPRRS MLV (isolate VR2332). Wesley et al. (1999) showed that the RFLP patterns change as the RespPRRS MLV spreads among a swine population. A glycine marker in the ORF5 gene of the vaccine virus is rapidly lost and replaced with arginine (Wesley et al., 1999). Since the herd 98-37120-2 was not vaccinated, it is possible that this herd is infected by a circulating acute PRRSV isolate. The RespPRRS MLV, which has been used in vaccination programs worldwide, has been incriminated as the origin of several recent Danish PRRSV isolates (Madsen et al., 1998). It has been reported that the

introduction of North American genotype of PRRSV in Denmark swine herds is due to the use and spread of the RespPRRS MLV (Madsen et al., 1998). Sequence and phylogenetic analyses indicated that the GP5 gene sequence of the acute PRRSV isolate 98-37120-2 is most closely related to those of Danish isolates of PRRSV that were confirmed to have been derived from RespPRRS MLV, suggesting the possible RespPRRS MLV origin of this acute PRRSV isolate. However, the data from this study do not fully support such a hypothesis that the emergence of acute PRRSV is due to reversion of MLVs to a pathogenic phenotype, as only one of the 8 acute isolates analyzed in the study was shown to be closely related to RespPRRS MLV. Clearly, future studies are warranted to determine if the acute PRRSV isolate 98-37120-2 can induce acute PRRS in specific-pathogen-free pigs under laboratory conditions.

Phylogenetic analysis of the ORF5 gene of available PRRSV isolates further confirmed the existence of two distinct genotypes of PRRSV: the European and North American genotypes. However, within the major North American genotype, numerous minor genotypes (or variants) were also observed. Goldberg et al. (2000) found that there is a lack of correlation between geographic proximity and genetic relatedness among North American PRRSV isolates. In this study, we found that the clustering of acute PRRSV isolates was also not associated with geographic origins. The ORF5 gene sequences of six acute PRRSV isolates from Iowa are more closely related to that of the North Carolina acute PRRSV isolate (92-95% nucleotide sequence identity) than to that of another Iowa acute isolate 98-37120-2 (88-89% nucleotide sequence identity). It is likely that the observed genetic heterogeneity among PRRSV isolates will continue to be the major obstacle to effectively prevent and control PRRS. The use of MLVs in herds

may lessen the clinical signs of PRRS following infection (Lager and Mengeling, 1997; Madsen et al., 1998; Mengeling et al., 1999a). However, the potential risk for reversion of MLVs to virulent phenotypes cannot be overlooked. By using a restriction-site marker that is present in the RespPRRS MLV VR2332, Mengeling et al. (1998) demonstrated that the marker was not detected in any of the 25 field isolates of PRRSV recovered before the use of the vaccine. However, the restriction-site marker was detected in 24 of 25 field isolates recovered after the introduction of the vaccine, and these field isolates were believed to be direct-line descendants of the vaccine (Mengeling et al., 1998). More importantly, these putative vaccine-related isolates produced more pronounced pathological changes than did the vaccine virus alone (Mengeling et al., 1998). In the absence of a new generation of vaccines, more studies are needed to fully evaluate the safety and efficacy of the current MLVs.

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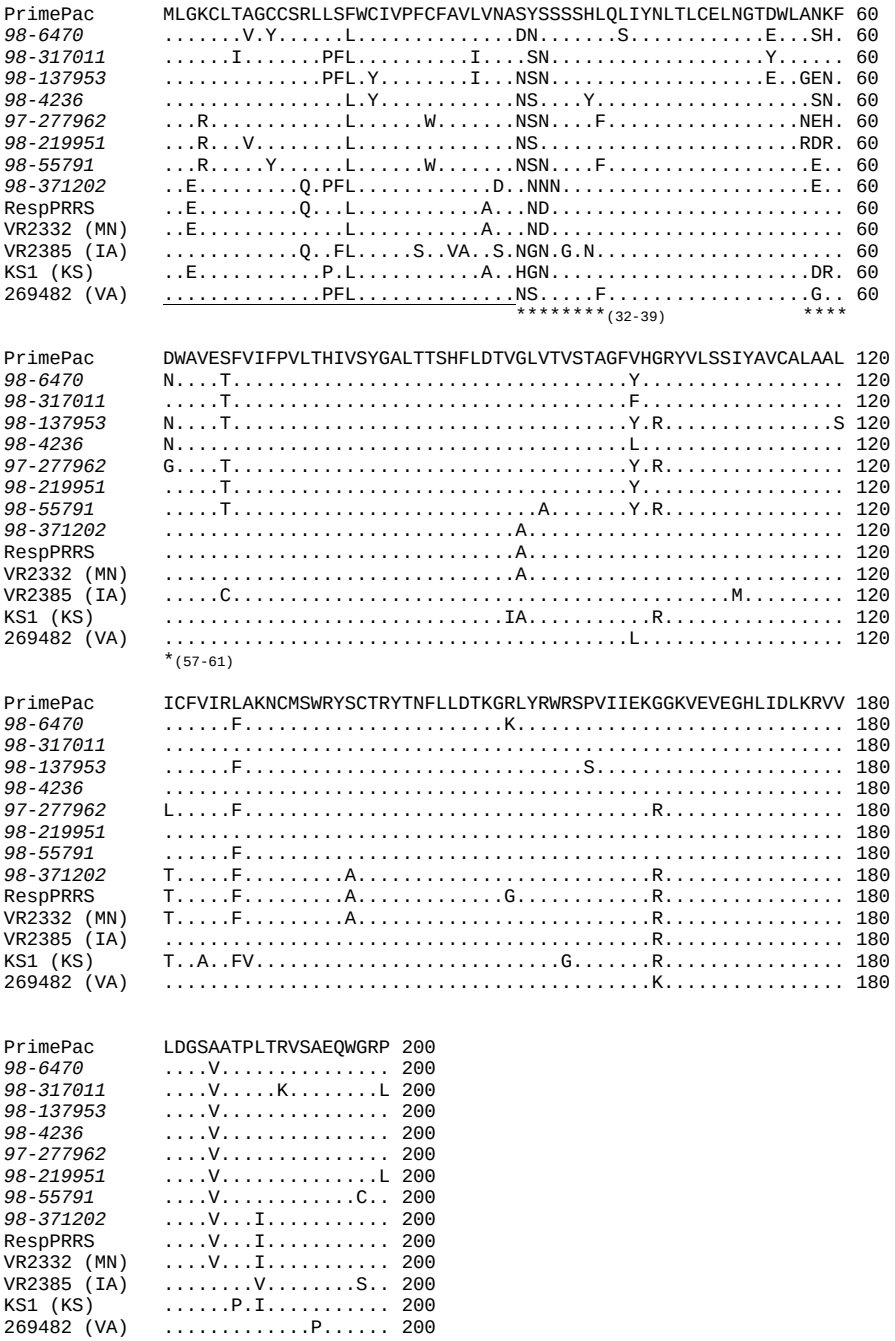

Fig. 2.1. Amino acid sequence alignment of the major envelope protein (GP5) of acute PRRSV isolates from this study, modified-live-vaccines (RespPRRS and PrimePac), and 4 selected PRRSV isolates identified prior to the acute PRRS outbreaks. The sequence of the PrimePac vaccine is shown on top, and only differences are indicated in other PRRSV isolates. The signal sequence is underlined. The two hypervariable regions are indicated by asterisks (*). The acute PRRSV isolates from this study are shown in italics. The GenBank database accession numbers for the sequences used in the alignment are listed in Table 1.

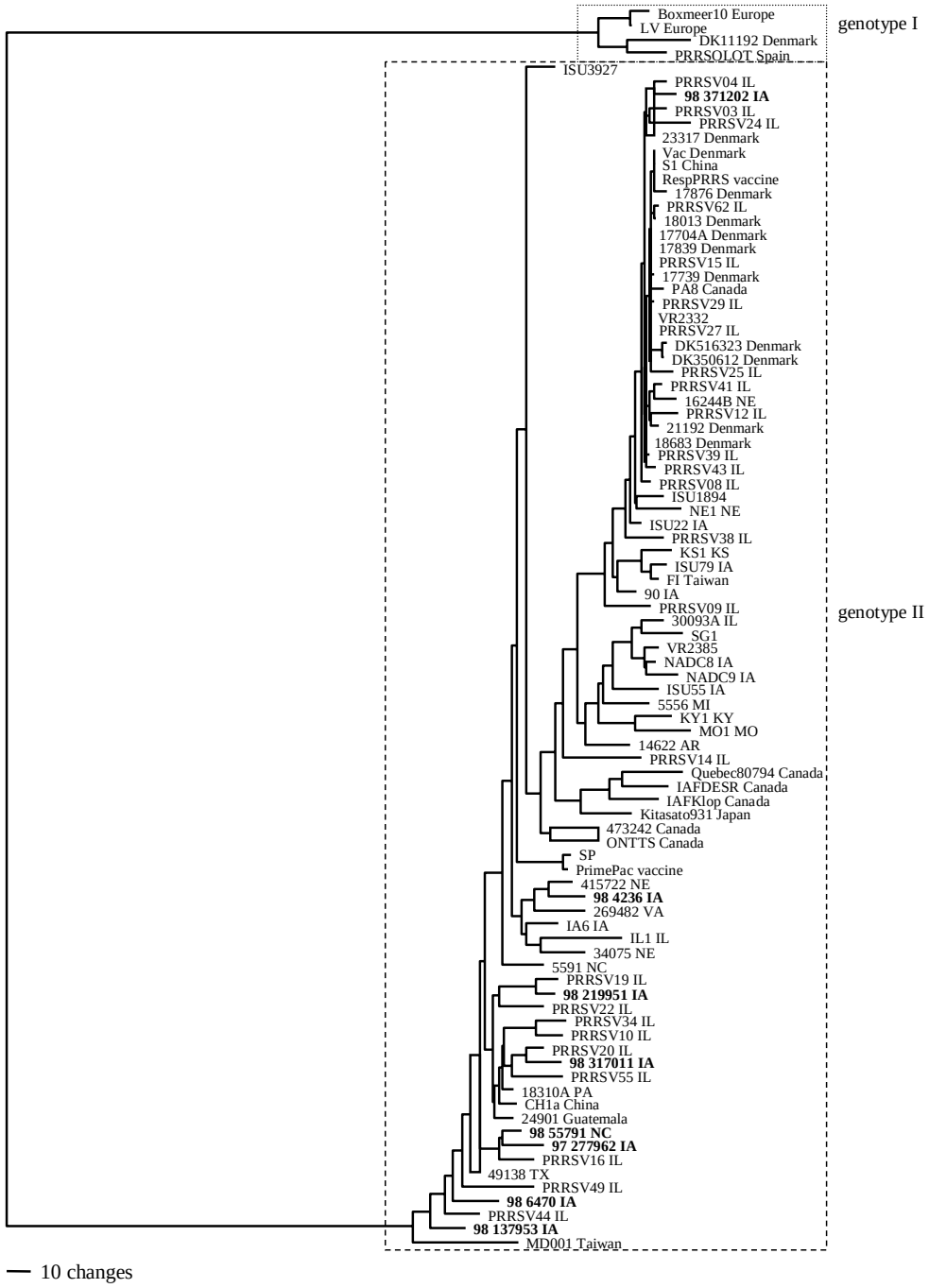

Fig. 2.2. A phylogenetic tree based on the nucleotide sequence of the ORF5 gene of PRRSV. The tree was constructed with the aid of the PAUP program. Heuristic searching with 1,000 replicates and midpoint rooting options was used to construct the tree. The scale bar representing the numbers of character state changes is shown. The eight acute isolates from this study are indicated in boldface. The GenBank database accession numbers for the sequences of PRRSV isolates used in the phylogenetic analyses are listed in Table 1.

Table 2.1. Acute PRRSV isolates and other known PRRSV isolates used in this study

Isolate ID	GenBank accession number	Herd Location	PRRSV vaccination
98-6470	AF339493	Iowa	RespPRRS
98-31701-1	AF339494	Iowa	RespPRRS and PrimePac
98-13795-3	AF339495	Iowa	RespPRRS
98-4236	AF339496	Iowa	RespPRRS
97-27796-2	AF339497	Iowa	RespPRRS and PrimePac
98-21995-1	AF339498	Iowa	RespPRRS and PrimePac
98-37120-2	AF339499	Iowa	No
98-21995-1	AF339500	North Carolina	RespPRRS
ISU22	U34297	Iowa	
ISU55	U34299	Iowa	
ISU79	U34300	Iowa	
ISU1894	U34296	Iowa	
ISU 3927	U34298	Iowa	
NADC8	U66394	Iowa	
NADC9	U66393	Iowa	
90	AF020049	Iowa	
IA6	L39362	Iowa	
VR2385	U03040	Iowa	
PRRSV03	AF176426	Illinois	
PRRSV04	AF176427	Illinois	
PRRSV08	AF176431	Illinois	
PRRSV09	AF176432	Illinois	
PRRSV10	AF176433	Illinois	
PRRSV12	AF176434	Illinois	
PRRSV14	AF176436	Illinois	
PRRSV15	AF176437	Illinois	
PRRSV16	AF176438	Illinois	
PRRSV19	AF176441	Illinois	
PRRSV20	AF176442	Illinois	
PRRSV22	AF176444	Illinois	
PRRSV24	AF176446	Illinois	
PRRSV25	AF176447	Illinois	
PRRSV27	AF176449	Illinois	
PRRSV29	AF176451	Illinois	
PRRSV34	AF176456	Illinois	
PRRSV38	AF176460	Illinois	
PRRSV39	AF176461	Illinois	
PRRSV41	AF176463	Illinois	
PRRSV43	AF176465	Illinois	
PRRSV44	AF176466	Illinois	
PRRSV49	AF176471	Illinois	
PRRSV55	AF176476	Illinois	
PRRSV62	AF176478	Illinois	
30093A	U66395	Illinois	
IL1	L39363	Illinois	
14622	U66399	Arkansas	
KS1	L39364	Kansas	
KY1	L39365	Kentucky	
5556	U66392	Michigan	
MO1	L39367	Missouri	
5591	U66379	North Carolina	
415722	U66386	Nebraska	
NE1	L39368	Nebraska	
16244B	AF046869	Nebraska	
34075	U66380	Nebraska	
18310A	U66381	Pennsylvania	
49138	U66382	Texas	
269482	U66385	Virginia	
PA8	AH006184	Canada	
Quebec80794	Z82995	Canada	
IAFDESR	U64930	Canada	
IAFKlop	U64928	Canada	
473242	U66387	Canada	
ONTTS	U64935	Canada	
S1	AF090173	China	
Ch1a	AF132118	China	
DK11192	AJ223078	Denmark	
23317	AF095518	Denmark	
Vac	AF095499	Denmark	
17876	AF095505	Denmark	
18013	AF095506	Denmark	
17704A	AF095500	Denmark	
17839	AF095503	Denmark	
17739	AF095519	Denmark	
DK516323	AJ223081	Denmark	
DK350612	AJ223079	Denmark	
21192	AF095517	Denmark	
18683	AF095513	Denmark	
Boxmeer 10	L04493	Europe	
LV	NC_002533	Europe	
24901	U66390	Guatemala	
Kitasato931	AB023782	Japan	
PRRSOLOT	X92942	Spain	
F1	AF030306	Taiwan	
MD001	AF035409	Taiwan	
RespPRRS vaccine	AJ223082	vaccine	
PrimePac vaccine	AF066384	vaccine	
VR2332	U87392	Minnesota	
SG1	L39369	US	
SP	AF184212	vaccine	

Table 2.2. Pairwise comparison of the nucleotide and amino acid sequences of the ORF5 gene of 8 acute PRRSV isolates from this study, European LV, PrimePac vaccine, Resp PRRS vaccine and its parent strain VR2332.

Isolate	98-6470 IA	98-317011 IA	98-137953 IA	98-4236 IA	97-277962 IA	98-219951 IA	98-371202 IA	98-55791 NC	LV Europe	RespPRRS vaccine	VR2332	PrimePac vaccine
98-6470 IA[a]		91[b]	94	92	91	91	88	93	62	88	89	91
98-317011 IA	90		92	92	90	92	89	92	62	90	90	92
98-137953 IA	91	92		92	93	91	89	94	62	89	89	91
98-4236 IA	93	91	92		91	92	89	92	63	90	90	92
97-277962 IA	92	90	93	92		91	89	95	63	89	90	91
98-219951 IA	93	92	91	94	93		89	92	62	90	90	90
98-371202 IA	88	90	88	89	89	88		89	62	97	97	91
98-55791 NC	92	91	92	92	96	93	89		63	89	90	92
LV Europe	56	54	54	56	57	57	56	56		62	63	61
RespPRRS vaccine	89	89	86	90	89	89	96	89	54		99	91
VR2332	90	90	87	91	90	90	96	90	54	99		92
PrimePac vaccine	92	93	89	94	91	94	91	92	55	93	94	

[a] The eight acute isolates sequenced in this study are shown in italics.

1 **[b] The values in the table represent % of nucleotide (upper right half) or amino acid (lower left half) sequence identities**

Chapter 3

Development of a heteroduplex mobility assay to identify field isolates of porcine reproductive and respiratory syndrome virus with nucleotide sequences closely related to modified live-attenuated vaccines

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) has been devastating the swine industry since the late 1980s. The disease has been controlled, to some extent, through the use of modified live-attenuated vaccines (MLVs) once available. However, such a practice periodically resulted in isolation or detection of vaccine-like viruses from pigs as determined by a partial genomic sequencing. In this study, we developed a heteroduplex mobility assay (HMA) for quickly identifying PRRSV isolates with significant nucleotide sequence identities (≥98%) with the MLVs. The major envelope gene (ORF5) of 51 PRRSV field isolates recovered before and after the introduction of the vaccines was amplified, denatured, and re-annealed with the respective HMA reference vaccine strains, Ingelvac® PRRS MLV and Ingelvac® PRRS ATP. Nine of the 51 field isolates and the VR2332 parent virus of Ingelvac® PRRS MLV, which were all highly related to Ingelvac® PRRS MLV with less than or equal to 2% nucleotide sequence divergence as determined by sequence analysis, were all identified by the HMA to form homoduplexes with the reference Ingelvac® PRRS MLV. No homoduplex-

forming field isolate was identified when Ingelvac® PRRS ATP was used as the HMA reference except for its parent virus JA142. Other field isolates with more than 2% nucleotide sequence divergence with the respective reference vaccine strain resulted in the formation of heteroduplexes with reduced mobility in polyacrylamide gel electrophoresis. The HMA results also correlated well with the results of phylogenetic analyses. The data indicated that the HMA developed in the study may be a rapid and efficient method for large-scale screening of potential vaccine-like PRRSV field isolates for further genetic characterization.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), often characterized by late-term abortions and stillbirths in sows and respiratory disease in nursery pigs, has resulted in extensive economic losses in the swine industry for over a decade (19). By estimation, the disease has caused approximate annual losses of $236 per inventoried adult female pig due to acute PRRS outbreaks (32).

Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of PRRS, is a small, enveloped, positive stranded RNA virus consisting of eight overlapping open reading frames (ORFs) (22, 26). The virus is genetically, antigenically, and pathogenetically heterogeneic (19). Substantial sequence divergence exists between the European and North American genotypes of the virus, sharing only about 70% nucleotide sequence identity (1, 11, 14-19, 23-26). Among the North American isolates, the PRRSV genomic sequences also vary significantly (10, 16-17, 25).

The heterogeneity of the virus has made it difficult to design effective vaccines based on a single PRRSV strain (19). Modified live-attenuated vaccines (MLVs) are currently used for the protection against PRRS mainly by providing protection against clinical disease (20, 31). These MLVs, such as Ingelvac® PRRS MLV, have reduced the incidence and severity of PRRS outbreaks on many farms. A severe form of PRRS, designated "acute PRRS" or "atypical PRRS", has recently been reported in the Midwestern U.S. Many of these acute PRRS outbreaks occurred in PRRSV MLV-vaccinated herds, suggesting that the commonly used MLVs are not fully effective. The occurrence of these acute PRRS in vaccinated pigs resulted in the recent introduction of another MLV, Ingelvac® PRRS ATP, to the market in February 2000.

Other concerns about the MLVs pertain to their safety. In Danish swine herds, Ingelvac® PRRS MLV vaccine virus has been shown to be capable of reverting to a pathogenic phenotype (5, 29, 33). Additionally, Mengeling et al. (21) confirmed that numerous vaccine-like field isolates, which contained the same restriction site marker that is found in the Ingelvac® PRRS MLV vaccine virus, were capable of causing disease more severe than any clinical signs induced by the MLV. The restriction site marker was not identified in any isolates collected prior to the introduction of the Ingelvac® PRRS MLV vaccine except for the parent strain ATCC VR2332. Other vaccine-like strains have also been reported (11, 19, 30) and some of these isolates were shown to be mildly to moderately pathogenic in pigs (30).

Due to the widespread use of Ingelvac® PRRS MLV and the periodic identification of vaccine-like isolates, there is a demand for a rapid assay that can be used routinely for identifying and differentiating these vaccine-like isolates from field isolates

of PRRSV. The current methods for differentiating PRRSV isolates include PCR amplification, subsequent sequencing and sequence analyses, or polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (35). Because these assays are costly and time-consuming, they are not very well suited for routine large-scale screening of viruses. The heteroduplex mobility assay (HMA) is a rapid and inexpensive method of differentiating viral isolates. Delwart et al. (8) originally developed the assay for genetic typing of HIV-1. More recently, HMA techniques have been applied to the study of other viruses such as influenza virus (38), feline immunodeficiency virus (2), measles virus (12), polio virus (7), Newcastle disease virus (4), and hepatitis C virus (9, 36). The assay relies on the formation of mismatched base pairs when two closely related DNA molecules are combined, denatured, and reannealed. The mismatches cause structural distortions in the newly formed DNA molecule resulting in heteroduplexes with reduced mobility on a polyacrylamide gel. It has been shown that the reduction in mobility is proportional to the degree of divergence between the two sequences (8). In this study, we report the development of a HMA and its use in the identification of PRRSV field isolates with more than 98% nucleotide sequence identities with the MLVs.

MATERIALS AND METHODS

Viruses. A total of 51 field isolates of PRRSV were analyzed by sequence and phylogenetic analyses and by HMA analyses using two reference strains, Ingelvac® PRRS MLV and Ingelvac® PRRS ATP. Ingelvac® PRRS MLV was introduced to the swine industry in early 1995, and Ingelvac® PRRS ATP was introduced to the swine

industry in February 2000 for use in the PRRS vaccination program. Twenty of the PRRSV isolates collected during PRRS outbreaks prior to the introduction of Ingelvac® PRRS MLV were obtained from Dr. Prem S. Paul's former laboratory at Iowa State University and from National Veterinary Service Laboratories (NVSL) in Ames, Iowa (Table 1). Fourteen PRRSV isolates were obtained from NVSL in Ames, Iowa from pigs in herds experiencing “acute PRRS" outbreaks in Iowa and North Carolina between 1997 and 1998 (11, Table 1). Fifteen PRRSV isolates were obtained from Iowa State University's Veterinary Diagnostic Lab (ISUVDL) (Table1) and some were determined by sequencing to be vaccine-like isolates. The two parent strains to the vaccines, ATCC VR2332 (Ingelvac® PRRS MLV) and JA142 (Ingelvac® PRRS ATP), were also included in this study.

Primer design. Two degenerate primers were designed to amplify the heterogeneic open reading frame (ORF) 5 of various PRRSV isolates. The primers were constructed based on a multiple sequence alignment of the ORF5 of 29 PRRSV isolates available in the GenBank database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, http://www.ncbi.nlm.nih.gov:80/Genbank/index.html). The sense primer AssayU (5'GTATGTTGGRGAAATGCTTGACC 3') began 2 bp upstream of the ORF5 start codon and includes the first 21 bp of the ORF5. The antisense primer AssayL (5'GGACGACCCCATTGTTCCGCTG3') consists of the 22 bp immediately preceding the last four bases at the 3' end of the ORF5 gene. The primers were synthesized and purified commercially (GIBCO-BRL, Gaithersburg, MD). The expected PCR product was 601 bp.

RNA extraction and RT-PCR. RNA was extracted with Trizol reagents (GIBCO-BRL) from 200 µl of each of the 51 PRRSV isolates and the 2 MLV reference strains. The total RNA was resuspended in 11.5 µl of DNase-free, RNase-free, and proteinase-free water. Reverse transcription was performed at 42^0 C for 60 min in the presence of a master mix consisting of 11.5 µl total RNA, 0.25 µl Superscript II reverse transcriptase, 1 µl 10 µM antisense primer, 0.5 µl of RNase inhibitor, 1 µl of 0.1M dithioteritol, 4 µl of 5x RT buffer, and 1 µl of 10 mM dNTPs. The resulting cDNA was amplified by PCR with the ORF5 sense and antisense primers and AmpliTaq Gold DNA polymerase (Perkin-Elmer, Shelton, CT). The PCR reaction parameters were 95^0 C for 9 min, followed by 39 cycles of 94^0 C for 1 min for denaturation, 50^0 C for 1 min for annealing, 72^0 C for 1.5 min for extension, followed by a final incubation period at 72^0 C for 7 min. The PCR products were examined on an agarose gel.

Sequencing and phylogenetic analyses. The PCR products were excised from an agarose gel and purified using the glassmilk procedure with a GENECLEAN kit (Bio101, Inc., Carlsbad, CA). The PCR products were sequenced at the Virginia Tech DNA Sequencing Facility with an automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA). The ORF5 genes of the 15 PRRSV isolates from ISUVDL were sequenced at the Iowa State University Nucleic Acid Facility as part of diagnostic investigation and the procedure for sequencing and sequence analysis is described elsewhere (6). The ORF5 sequences of the two vaccines and their parent strains and 11 field isolates of PRRSV have been reported previously (11, 15-18, 23-25). The ORF5

sequences of the remaining 38 PRRSV isolates sequenced in this study were deposited in GenBank database under accession numbers AF537919 to AF537956. The sequences of all isolates were analyzed using the MacVector program (Oxford Molecular, Inc., Oxford, England).

The ORF5 genes of the 51 field isolates and the reference strains were phylogenetically analyzed using the PAUP program (David L. Swofford, Smithsonian Institute, Washington, DC, distributed by Sinauer Associates, Inc., Sunderland, MA) to create a consensus tree. Lelystad virus (22) was also included as a representative of European genotype of PRRSV in the phylogenetic analysis.

HMA. Five µl of the PCR product from each reference virus was combined with 5 µl of PCR product from each of the 51 PRRSV field isolates and 1.1 µl of annealing buffer (1M NaCL, 100 mM Tris pH 7.8, 20 mM EDTA) in a 0.2 ml thin-walled polypropylene tube (Perkin-Elmer, Shelton, CT). The mixture was denatured at 95^0 C for 5 min and reannealed at 50^0 C for 30 min in a thermocycler. Heteroduplexes and homoduplexes were separated on an 8.0% nondenaturing polyacrylamide gel at 150 V for 5 hours in TBE buffer. The gels were stained with ethidium bromide and visualized under UV light. Homoduplexes were identified as a single band, while more than one band with reduced mobility was classified as a heteroduplex.

RESULTS

Sequence analysis. The ORF5 genes of all 51 PRRSV isolates and the two MLV reference strains were successfully amplified using the degenerate primers AssayU and

AssayL. The amplified ORF5 regions of the isolates were sequenced. The sequences were compared to determine the percent nucleotide sequence identities with the corresponding sequence of each reference strain. Sequence analyses revealed that the field isolates shared 88-99% identity with Ingelvac® PRRS MLV and 87-96% identity with the Ingelvac PRRS ATP (Table 1). In general, the mismatches seemed to occur throughout the ORF5 in both reference strains. The isolates ISU4 and ISU 3927 contained a 3-nucleotide deletion at nucleotide positions 76-78 when compared to both reference strains. Ingelvac® PRRS MLV and Ingelvac® PRRS ATP shared 99% nucleotide sequence identity with their respective parent strains, VR2332 and JA142. Ingelvac® PRRS MLV and VR2332 differed by 2 nucleotides while Ingelvac® PRRS ATP and JA142 differed by 5 nucleotides in the amplified region.

HMA using Ingelvac® PRRS MLV as the reference. When Ingelvac® PRRS MLV was used as the reference for the HMA, ten of the 51 PRRSV isolates formed a homoduplex and the remaining ones formed heteroduplexes (Table 1). The 10 homoduplex-forming isolates displayed 98-99% nucleotide sequence identity with Ingelvac® PRRS MLV. Eight of the ten homoduplex-forming isolates were isolated after the introduction of the Ingelvac® PRRS MLV vaccine to the swine industry. The two remaining homoduplex-forming isolates included isolate VR2332, which is the parent strain of Ingelvac® PRRS MLV, and isolate 93-41462 that was isolated from outbreaks occurring prior to the introduction of Ingelvac® PRRS MLV. The remaining isolates formed heteroduplexes with reduced mobility of variable degrees when compared

to the reference. Based on surveys of the polyacrylamide gels of all of the isolates, the reduction in mobility is roughly proportional to the level of divergence between the sequences (data not shown). However, due to the presence of unpaired nucleotides at positions 76-78, the isolates ISU4 and ISU 3927 displayed an exaggerated reduction in mobility as compared to the other isolates with the same 91% nucleotide sequence identity (Fig. 1A, lanes 8, 9). This increased reduction in mobility due to deletions, which has been described in other HMA studies (8), was also observed in the HMA with Ingelvac® PRRS ATP vaccine as the reference.

HMA using Ingelvac® PRRS ATP vaccine as the reference. When Ingelvac® PRRS ATP vaccine strain was used as the reference, fifty of the 51 isolates formed a heteroduplex with the reference strain. The parent strain to the vaccine, JA142, formed a near homoduplex with the reference. The band formed between Ingelvac® PRRS ATP and its parent strain JA142 migrated only slightly slower than the homoduplex formed when Ingelvac® PRRS ATP was combined, denatured, and re-annealed with itself (Fig. 1B, lanes 1, 2). Sequence analysis demonstrates five mismatched base pairs between the amplified regions of the Ingelvac® PRRS ATP vaccine and its parental strain JA142. These mismatches, occurring at positions 103, 169, 217, 273, and 341, locate towards the middle of the ORF5 region. The remaining heteroduplex-forming isolates demonstrate varying degrees of reduced mobility, which appear to correlate with the degree of divergence between the sequences. All of the isolates used in the study were isolated prior to the introduction of the Ingelvac® PRRS ATP vaccine.

Phylogenetic analysis. A phylogenetic tree was constructed based on the two reference strains Ingelvac® PRRS MLV and Ingelvac® PRRS ATP, the 51 PRRSV field isolates including the two vaccine parental strains, and the European Lelystad virus. As illustrated in figure 2, the phylogram demonstrates the distinct European and North American genotypes among PRRSV isolates and that the isolates analyzed in this study all belong to the North American genotype. The phylogenetic analysis also demonstrates that the North America isolates form several genotypic clusters, indicating the heterogeneity of the virus (Fig. 2, Table 1). All the 10 isolates sharing ≥98% nucleotide sequence identities and forming homoduplexes in the HMA with Ingelvac® PRRS MLV clustered together in the phylogenetic tree with the Ingelvac® PRRS MLV. Similarly, the JA142 strain forming a near homoduplex in the HMA and sharing 99% sequence identity with Ingelvac® PRRS ATP clustered together with Ingelvac® PRRS ATP (Fig. 2).

DISCUSSION

Ingelvac® PRRS MLV has been used as a tool for preventing and/or controlling PRRS since early 1995. Recently, numerous vaccine-like PRRSV isolates have been identified from non-symptomatic persistently infected pigs and diseased animals. In 1996, Danish sow herds experienced clinical signs of PRRS after having been vaccinated in an extra-label fashion with Ingelvac® PRRS MLV a few months prior. Through the use of monoclonal antibodies, the virus causing the outbreaks was found to be closely related to the North American phenotype. Further genetic studies confirmed that the

isolates from Danish swine herds were of Ingelvac® PRRS MLV vaccine origin, indicating that the vaccine virus is capable of spreading and possibly reverting to a pathogenic phenotype (5, 13, 28, 30, 33). Other North American field isolates of PRRSV have also been speculated to be vaccine-related (11, 21) based on genetic analyses of the ORF5 genes, and some were found to cause mild-to-moderate disease in pigs (30).

In this study, a heteroduplex mobility assay was developed to rapidly screen field isolates of PRRSV for potential vaccine-like isolates. The HMA developed in this study uses the heterogeneic ORF5 gene (6, 10, 19) as a focus for identifying the genetic divergence between the isolates. The ORF5 gene encodes the major envelope protein of PRRSV, which is exposed outside the virions and under immune selection pressure. Fifty-one PRRSV field isolates collected from various PRRS outbreaks were included in the study to evaluate the HMA. Sequence analyses revealed that nine of these isolates (93-41462, ISUVDL2001027011, ISUVDL2001044010, ISUVDL2001047450, ISUVDL2001016727, ISUVDL2001018571A, ISUVDL2001019139, ISUVDL2001021373A, and ISUVDL2001018087B) shared ≥98% nucleotide sequence identity with Ingelvac® PRRS MLV. The VR2332 isolate with 99% sequence identity with the Ingelvac® PRRS MLV is the parent strain of Ingelvac® PRRS MLV vaccine. It has been shown that low levels of sequence divergence without insertions or deletions are generally associated with homoduplex formation in the HMA (3, 8). In this study, each of the nine field isolates with ≥98% sequence identity with the Ingelvac® PRRS MLV and the VR2332 parent virus of the Ingelvac® PRRS MLV formed a homoduplex in the HMA when combined, denatured, and re-annealed with the reference strain Ingelvac® PRRS MLV vaccine.

By analyzing the HMA results, the sequence data, and the time of virus isolation, we speculated on the origins of these homoduplex-forming isolates that shared more than 98% nucleotide sequence identities with the Ingelvac® PRRS MLV. The isolates ISUVDL2001027011, ISUVDL2001044010, ISUVDL2001047450, ISUVDL2001016727, ISUVDL2001018571A, ISUVDL2001019139, ISUVDL2001021373A, and ISUVDL2001018087B were all isolated after the introduction of the Ingelvac® PRRS MLV. Wesley et al. (35) showed that a glycine residue at position 151 of the ORF5 is rapidly substituted for an arginine upon replication in pigs, suggesting that arginine at position 151 could be a marker for cell culture adaptation of PRRSV. Notably, five of the 9 homoduplex-forming field isolates contain an arginine residue at position 151, suggesting that they may be of Ingelvac® PRRS MLV vaccine origin. Two isolates (ISUVDL2001019139, ISUVDL2001018087B) each had an isoleucine residue, one isolate (93-41462) had a valine residue, and one isolate (ISUVDL2001016727) retained the glycine residue at position 151. The other substitutions in the sequences tend to locate at the signal sequence (amino acids positions 1-31) and the hypervariable region (amino acids positions 32-39) near the N-terminus. The isolate 93-41462, which was isolated prior to the use of the Ingelvac® PRRS MLV and had 99% nucleotide sequence identity with both Ingelvac® PRRS MLV and its parent virus VR2332, is more likely to be derived from the wild-type VR2332 instead of Ingelvac® PRRS MLV. The origins of the remaining three homoduplex-forming isolates (ISUVDL2001019139, ISUVDL2001018087B and ISUVDL2001016727) are not known. Seven of the 9 isolates with 96 to 97% nucleotide sequence identities with the Ingelvac® PRRS MLV contain an arginine residue at position 151, and the remaining two isolates

both contain a lysine residue. Due to the nature of rapid genetic changes of the virus in pigs (6, 37), it is logical to speculate that these isolates with more than 96% nucleotide sequence identities with Ingelvac® PRRS MLV could be derived from Ingelvac® PRRS MLV due to its widespread use in the vaccination program or from its parent virus, ATCC VR2332, which is likely still circulating in the pig population. However, a definitive conclusion as to the origins of these vaccine-like isolates can not be drawn without determining the complete genomic sequence of these isolates.

Ingelvac® PRRS ATP vaccine was introduced to swine industry only in February 2000, thus vaccine-like field isolates due to the spread of ATP vaccine occur less frequently. Except for isolate JA142 which is the parent virus of the Ingelvac® PRRS ATP vaccine, all other field isolates shared less than 97% nucleotide sequence identity, and formed heteroduplexes with the Ingelvac® PRRS ATP vaccine reference. The isolate JA142 formed a near homoduplex with the reference strain. At 99% nucleotide sequence identity with the reference Ingelvac® PRRS ATP vaccine, JA142 contained 5 centrally located mismatches with the vaccine virus. The significance of location, as well as the quantity, of mismatches has been noted in previous HMA studies which demonstrated that the clustering of mismatches toward the center of a fragment can have a more exaggerated effect on the mobility reduction (27, 34). We speculate that concentration of the 5 mismatches toward the center of the ORF5 explains the slight reduction in mobility of the JA142 even at only 1% divergence with the reference vaccine virus. A similar phenomenon was not observed between Ingelvac® PRRS MLV and its parent strain VR2332 because these isolates had only two mismatches located at the

periphery of the ORF5 gene. The other isolates with 1% divergence also tended to have mismatches found mainly at the 5' end of the ORF5.

The results of phylogenetic analyses of the 51 field isolates agreed well with both the sequence results and the HMA data. All the homoduplex-forming isolates with ≥98% sequence identity to the Ingelvac® PRRS MLV vaccine are phylogenetically closely related to the Ingelvac® PRRS MLV. The Ingelvac® PRRS ATP vaccine and its parent strain JA142 are also clustered together in the phylogenetic tree. Genotypic relationships between some of the closely related PRRSV isolates were also clearly identifiable in this study. The group of acute PRRSV isolates, 98-13795-1, 98-13795-3, 98-13795-5, 98-13795-10, 98-13795-13, were all isolated from the same herd outbreak in 1998 and they share 99-100% nucleotide sequence identity with each other and clustered together in the phylogenetic tree.

The HMA assay developed in this study correlated well with both sequence and phylogenetic analyses results, and correctly identified all field isolates with ≥98% nucleotide sequence identities with the MLVs. Therefore, the HMA developed in this study defines viruses of ≥98% nucleotide sequence identity with the MLVs. This HMA will be a useful and more efficient method for large-scale screening of potential vaccine-like isolates for further genetic characterization. Using this assay, a field isolate of PRRSV within 2% sequence divergence in the ORF5 gene of the commonly used Ingelvac® PRRS MLV vaccine, can be readily identified. The assay is also an effective method for identifying closely related PRRSV isolates through the formation of similar or identical heteroduplex banding patterns when combined with any reference strain.

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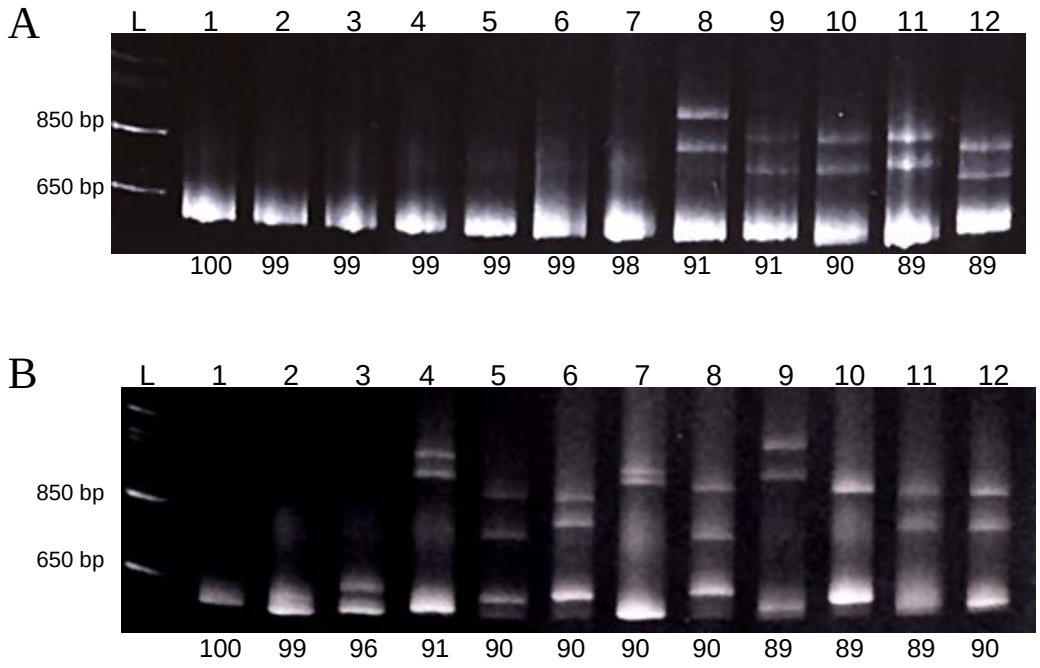

Figure 3.1.

A. HMA results of selected PRRSV isolates when Ingelvac® PRRS MLV vaccine was used as the reference. L: 1 kb DNA ladder; lane 1: reference + reference; lane 2: reference + VR2332; lane 3: reference + 16727-01; lane 4: reference + 44010A-01; lane 5: reference + 47450-01; lane 6: reference + 27011-01; lane 7: reference + 18087B-01; lane 8: reference + ISU3927; lane 9: reference + ISU55; lane 10: reference + JA142; lane 11: reference + ATP; lane 12: reference + 4518-01. Percent identity of nucleotide sequence between reference and respective PRRSV isolates is listed below each lane.

B. HMA results of selected PRRSV isolates when Ingelvac® PRRS ATP vaccine was used as the reference. L: 1 kb DNA ladder; lane 1: reference + reference; lane 2: reference + JA142; lane 3: reference + 97-27796-4; lane 4: reference + ISU3927; lane 5: reference + ISU28; lane 6: reference + ISU30262; lane 7: reference + 98-4236; lane 8: reference + 98-13795-3; lane 9: reference + 93-36048; lane 10: reference + 93-4506; lane 11: reference + PRRS; lane 12: reference + VR2332. Percent identity of nucleotide sequence between reference and respective PRRSV isolates is listed below each lane.

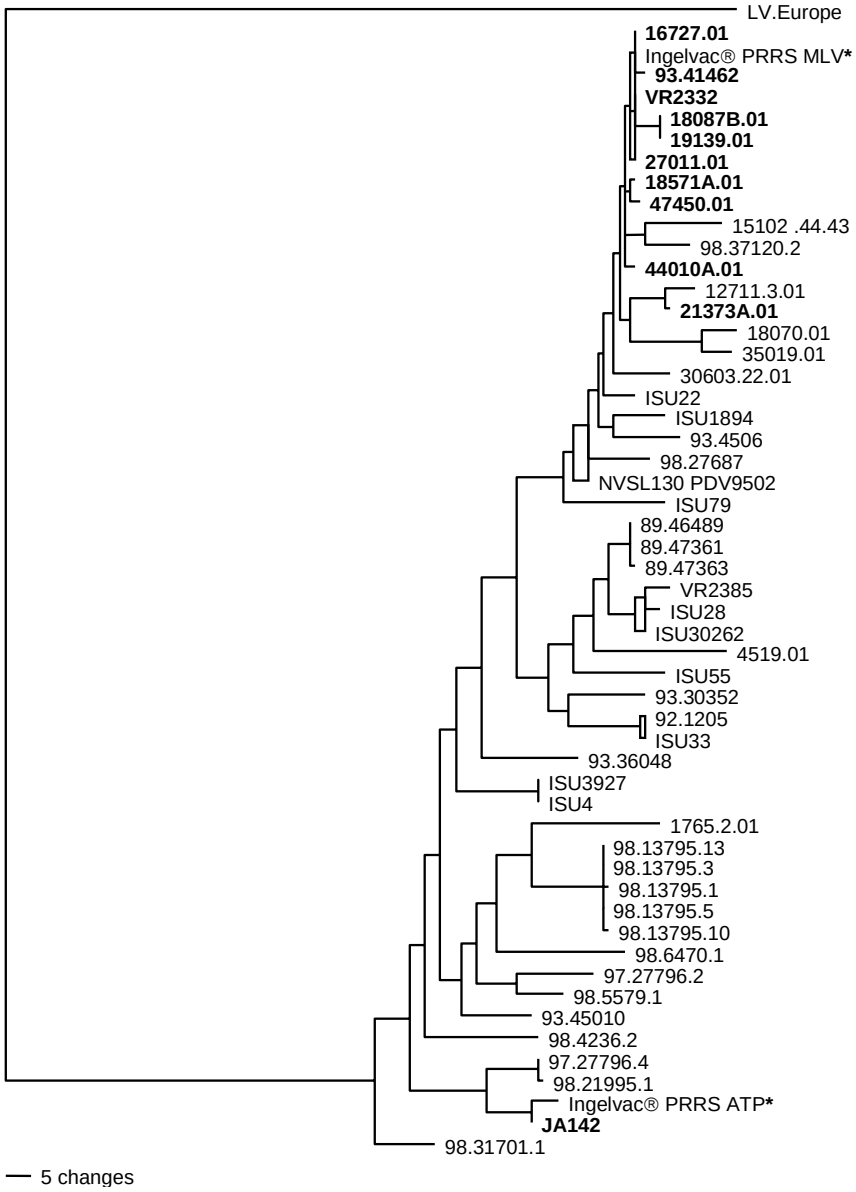

Figure 3.2. A phylogenetic tree based on the ORF5 region of PRRSV isolates analyzed in the study: two MLV vaccines (Ingelvac® PRRS MLV and Ingelvac® PRRS ATP, designated with a *) used as the HMA references, 51 PRRSV field isolates analyzed in the study including the parent strains of the two vaccines, VR2332 and JA142, and the European LV as the outgroup. Homoduplex-forming isolates are shown in **boldface**. The tree was constructed with the PAUP program using a heuristic search option with midpoint rooting and 1,000 replicates.

Table 3.1. Correlation between the percentage of nucleotide sequence identity of PRRSV isolates and the heteroduplex mobility assay (HMA) results when Ingelvac® PRRS MLV and Ingelvac® PRRS ATP vaccines were used, respectively, as the reference strains for the HMA

Isolate #	Lab Origin [a]	PRRS MLV			PRRS ATP		
		% nucleotide sequence identity	homoduplex(+) or heteroduplex(-)	After vaccine introduced (Y/N)	% nucleotide sequence identity	homoduplex(+) or heteroduplex(-)	after vaccine introduced (Y/N)
PRRS MLV	BIVI	100	+		89	-	N
VR2332	BIVI	99	+	N	90	-	N
27011-01	ISUVDL	99	+	Y	89	-	N
44010-01	ISUVDL	99	+	Y	90	-	N
47450-01	ISUVDL	99	+	Y	90	-	N
16727-01	ISUVDL	99	+	Y	89	-	N
18571A-01	ISUVDL	99	+	Y	89	-	N
93-41462	NVSL	99	+	N	89	-	N
19139-01	ISUVDL	98	+	Y	89	-	N
21373A-01	ISUVDL	98	+	Y	89	-	N
18087B-01	ISUVDL	98	+	Y	89	-	N
30603-22-01	ISUVDL	97	-	Y	88	-	N
12711-3-01	ISUVDL	97	-	Y	89	-	N
ISU22	ISUPSP	97	-	N	89	-	N
PDV9502	NVSL	97	-	N	91	-	N
98-37120-2	*NVSL	97	-	Y	89	-	N
15102 44.43	ISUVDL	96	-	Y	88	-	N
ISU1894	ISUPSP	96	-	N	89	-	N
93-4506	NVSL	96	-	N	89	-	N
98-27687	*NVSL	96	-	Y	89	-	N
35019-01	ISUVDL	95	-	Y	88	-	N
18070-01	ISUVDL	95	-	Y	87	-	N
ISU79	ISUPSP	94	-	N	88	-	N
ISU30262	ISUPSP	93	-	N	90	-	N
89-47361	NVSL	93	-	N	90	-	N
89-46489	NVSL	93	-	N	90	-	N
89-47363	NVSL	93	-	N	90	-	N
ISU28	ISUPSP	92	-	N	90	-	N
93-30352	NVSL	92	-	N	92	-	N
1765-2-01	ISUVDL	91	-	Y	88	-	N
ISU 3927	ISUPSP	91	-	N	91	-	N
ISU33	ISUPSP	91	-	N	88	-	N
92-1205	NVSL	91	-	N	87	-	N
93-36048	NVSL	91	-	N	89	-	N
93-45010	NVSL	91	-	N	91	-	N
ISU55	ISUPSP	91	-	N	89	-	N
ISU4	ISUPSP	91	-	N	91	-	N
98-21995-1	*NVSL	90	-	Y	95	-	N
98-31701	*NVSL	90	-	Y	91	-	N
97-27796-4	*NVSL	90	-	Y	96	-	N
JA142	BIVI	90	-	Y	99	+	N
PRRS ATP	BIVI	89	-	Y	100	+	
4519-01	ISUVDL	89	-	Y	88	-	N
97-27796-2	*NVSL	89	-	Y	90	-	N
98-5579	*NVSL	89	-	Y	91	-	N
98-4236	*NVSL	89	-	Y	90	-	N
VR2385	ISUPSP	89	-	N	89	-	N
98-13795-3	*NVSL	88	-	Y	90	-	N
98-6470	*NVSL	88	-	Y	89	-	N
98-13795-1	*NVSL	88	-	Y	89	-	N
98-13795-5	*NVSL	88	-	Y	90	-	N
98-13795-10	*NVSL	88	-	Y	90	-	N
98-13795-13	*NVSL	88	-	Y	90	-	N

Chapter 4

Direct inoculation of RNA transcripts from an infectious cDNA clone of porcine reproductive and respiratory syndrome virus (PRRSV) into the lymph nodes and tonsils of pigs initiates PRRSV infection *in vivo*

K.F. Key, J. DiCristina, J. Gillespie, D.K. Guenette, and X.J. Meng

ABSTRACT

The recent construction of PRRSV infectious cDNA clones affords the opportunity for structural and functional studies of PRRSV genes. However, the inherited instability of PRRSV genome, the requirement of cell culture propagation, and poor virus recovery have limited the usefulness of PRRSV reverse genetics system for *in vivo* studies. Here we report a unique strategy of infecting pigs by bypassing the traditional *in vitro* cell culture step required for *in vivo* studies. We demonstrated that inoculation of RNA transcripts of a PRRSV infectious cDNA clone directly into the lymph nodes and tonsils of pigs produces active PRRSV infection. The information from this study will have significant implications for the study of the molecular mechanism of PRRSV pathogenesis using the reverse genetics system.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is arguably the most important swine pathogen causing a devastating disease, PRRS, in pigs worldwide

(14, 16, 20). PRRSV, a single-strand positive-sense RNA genome of approximately 15 kb in size, belongs to the family *Arteriviridae* in the Order *Nidovirales* (3, 15, 18-19). Despite intensive research efforts on PRRSV in the last decade, PRRS remains difficult to control. The underlying molecular mechanism of PRRSV pathogenesis, especially the mechanism of PRRSV persistence (1, 26), remains unknown. The heterogeneic nature of PRRSV due to quasispecies evolution, inherited high mutation rates, and potential recombination render the current vaccines less effective (4, 8, 23). Consequently, variant strains of PRRSV continue to emerge and cause diseases in vaccinated herds in the field (6, 9, 16-17, 22). Understanding the basic biology and molecular mechanism of pathogenesis of the virus will be the key for the rational design of future generations of vaccines against PRRSV.

The recent successful constructions of PRRSV infectious cDNA clones (5, 17, 21, 25, 28) provided the necessary tools to study the structural and functional relationship of PRRSV genes and to delineate the molecular mechanisms of PRRSV replication and pathogenesis. Unfortunately, virus recovery from PRRSV infectious cDNA clones in the current cell culture system is often problematic, and sometime requires the use of two different cell lines (19, 21). Consequently, the recovered virus from the PRRSV infectious clone can have unwanted mutations due to cell culture propagation, which thus limits its use for subsequent *in vivo* functional studies. In this study, we developed a unique procedure to successfully infect pigs by directly inoculating RNA transcripts from a PRRSV infectious cDNA clone into the lymph nodes and tonsils of pigs, thus bypassing the *in vitro* cell culture step. We demonstrated that RNA transcripts derived from a North American PRRSV infectious cDNA clone are viable and infectious when directly

injected into the lymph nodes and tonsils of pigs, the sites of PRRSV replication *in vivo* (24).

MATERIALS AND METHODS

To generate RNA transcripts of PRRSV, the PRRSV infectious cDNA clone pFL12 (25) was linearized by digestion with restriction enzyme AclI. The linearized plasmid DNA was extracted with phenol-chloroform and precipitated with ethanol. Full-length RNA transcripts of PRRSV genome were generated using the mMESSAGE mMACHINE kit (Ambion, Inc., Austin, TX) in ten 100 µl reactions each containing 50 µl 2X NTP/CAP (15mM ATP, 15mM CTP, 15mM UTP, 3mM GTP, 12mM cap analog), 10 µl 10X reaction buffer, 5 µl of 30mM GTP, 6 µg (in 25 µl) of template linearized plasmid DNA, and 10 µl (200 units/µl) of T7 polymerase (7). Each reaction was incubated at 37° C for 2 hrs. The reaction mixtures were pooled and immediately frozen on dry ice and injected into the lymph nodes and tonsils of pigs within 3 hrs. A 2-µl aliquot of the pooled reaction mixtures was examined by agarose gel electrophoresis to check for the RNA quality and to estimate RNA concentration.

To test the infectivity of the *in vitro* RNA transcripts, six conventional pigs of approximately 7 weeks of age were divided into two groups of 4 and 2 each. All pigs were negative for PRRSV antibody prior to the inoculation. Group 1 consisted of 4 pigs (pig ID numbers 190, 191, 192, 193), and each pig was inoculated with equal amounts of the pooled RNA transcription mixtures (250 µl of the transcription mixture diluted in 350 µl of sterile, ice cold phosphate buffered saline) directly into both the superficial inguinal lymph nodes and the palatine tonsils (150 µl in each lymph node, and 150 µl in each

tonsil). The 2 pigs in group 2 (pig ID numbers 177, and 178) were each mock inoculated similarly with phosphate buffered saline (PBS) in the same sites as negative controls. The animal study was approved by Virginia Tech IACUC, and the animals were housed under conditions that met IACUC requirement. The pigs were restrained manually for inoculation. Serum samples were collected prior to inoculation and weekly thereafter from each pig until 63 days post inoculation (dpi). Tonsil biopsies were collected from each pig on dpi 21, 42, and 63. For tonsil biopsies, the pigs were chemically restrained with 11mg/kg Ketamine administered intramuscularly ten minutes prior to the procedure and 2% topical lidocaine applied to the palatine tonsils 3 minutes prior to the biopsy. The samples were collected using a dowel and dermal punch biopsies while the mouth was held open with a large animal mouth speculum.

RESULTS AND DISCUSSION

The pigs in group 1 experienced inappetence and lethargy during the first week post-inoculation while the group 2 control pigs exhibited no clinical signs of PRRSV infection throughout the study. Serial serum samples were tested by an ELISA for PRRSV-specific antibodies at Iowa State University Veterinary Diagnostic Laboratory (Fig. 1). Group 1 pigs all seroconverted to PRRSV-specific antibodies during the first two weeks postinoculation (Fig. 1) and remained seropositive for the duration of the study, indicating that group 1 pigs were successfully infected by PRRSV. The control pigs in group 2 remained seronegative throughout the study.

All sera and tonsil samples were tested for the presence of PRRSV RNA by a RT-PCR. Primer ORF7U (5’-GGGGAATGGCCAGCCAGTCAATCAGC -3’) and primer

ORF7L (5'-GTATGATGCGTCGGCAAACTAAACTCC-3') were designed based on the published sequence of pFL12 parent strain 977895 (25) to amplify a 294-bp fragment in ORF7. RNA was extracted with TriZol Reagent (Molecular Research Center, Cincinnati, OH) from 100 µl of sera or tonsil tissue homogenates. Reverse transcription was carried out at 42°C for 1 hr in a 19.5 µl reaction containing 0.5 µl RNasin, 1 µl DTT (0.1 M), 1 µl dNTPs (10mM), 0.5 µl reverse transcriptase, 4 µl 5X RT buffer, 1 µl ORF7L primer, and 11.5 µl RNA. The resulting cDNA was amplified by PCR with the following parameters: an initial denaturation step at 95°C for 9 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 2.5 min, and followed by a final incubation at 72°C for 7 min. The PCR products were examined by electrophoresis on a 0.8% agarose gel.

All of the sera samples from the group 1 pigs collected on dpi 7 and two of the sera samples (pig #191 and #192) on dpi 14 were positive by RT-PCR, further confirming that the group 1 pigs were all infected by PRRSV. The remaining sera samples from group 1 pigs were negative. None of the sera samples from group 2 negative control pigs was positive by RT-PCR and all of the tonsil tissues collected at dpi 21, 42, and 63 from both groups of pigs were negative by RT-PCR. The amplified RT-PCR products from group 1 pigs were subsequently sequenced. Sequence analyses revealed that the virus recovered from infected pigs in group 1 originated from the inoculum (data not shown), which was the RNA transcript from pFL12 infectious cDNA clone of PRRSV. In addition, a serum sample collected from one of the group 1 pigs (pig #190) at dpi 7 was inoculated onto a monolayer of MARC-145 cells for virus isolation. Evidence of virus replication was observed in inoculated MARC-145 cells as

immunoflorescence assay (IFA) with a PRRSV-specific monoclonal antibody (SDOW-17) detected PRRSV antigens in the infected cells at 3 dpi (data not shown), further confirming that infectious virus was produced in the infected animals.

The availability of PRRSV infectious cDNA clones now affords us the opportunity to genetically manipulate the PRRSV genome *in vitro*, and thus allows the recovered virus, with distinct genetic markers from the *in vitro* reverse genetics system, to be functionally studied in pigs. Therefore, even a few unwanted mutations in the genome of the recovered PRRSV *in vitro* can have a significant impact when evaluating the specific characteristics of the engineered virus such as replication and pathogenesis *in vivo*. The unique method, known as *in vivo* transfection, developed in this study bypasses the intermediate *in vitro* steps such as *in vitro* transfection, virus propagation, and cell culture passaging previously required for *in vivo* studies thereby eliminating the likelihood of unwanted mutations introduced during these intermediate *in vitro* steps. The *in vivo* transfection technique is based on the independent infectivity of naked positive-stranded RNA viral genomes, and has been successfully used in the studies of other positive-stranded RNA viruses such as hepatitis C virus (27), hepatitis E virus (7), foot and mouth disease virus (2), and rabbit hemorrhagic diseases virus (13). To our knowledge, this is the first report of successful *in vivo* transfection of animals with a RNA viral genome of more than 10 kb. The *in vivo* transfection system for PRRSV developed in this study offers a more direct and much simpler and faster method for recovering infectious PRRSV from cDNA clones by bypassing the traditional cell cultures, and will facilitate future molecular mechanistic studies of PRRSV pathogenesis and replication in pigs.

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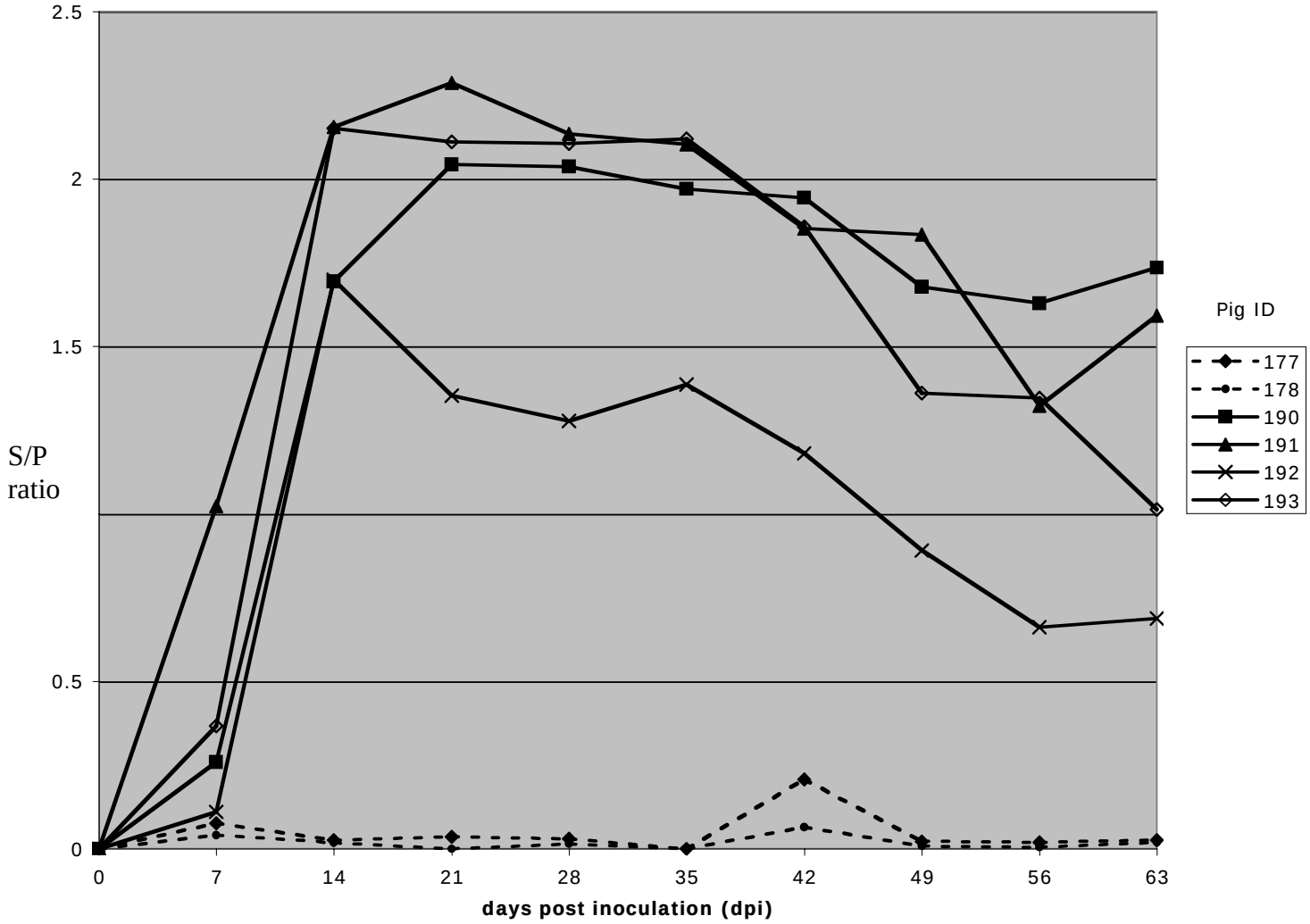

Fig. 4.1. Antibody response to PRRSV in pigs intralymphoid- and intratonsillar-inoculated with RNA transcripts from an infectious cDNA clone of PRRSV (pig ID numbers 190, 191, 192, and 193) or with PBS buffer (pig ID numbers 177 and 178). The Y-axis is the S/P ratio of the ELISA OD values, and the X-axis is the days postinoculation (DPI).

Chapter 5

Evaluation of quasispecies evolution of porcine reproductive and respiratory syndrome virus (PRRSV) in pigs during an acute infection resulting from *in vivo* transfection of pigs with RNA transcripts from an infectious cDNA clone of PRRSV

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ABSTRACT

Since the mid 1980s, porcine reproductive and respiratory syndrome (PRRS) has devastated swine populations worldwide. Porcine reproductive and respiratory syndrome virus (PRRSV), a positive single-stranded RNA virus, is responsible for PRRS outbreaks. Like many other RNA viruses, PRRSV exists as a quasispecies population, or a population of closely related mutant and recombinant genomes that are variants of one dominant sequence. Earlier studies of PRRSV quasispecies have often begun with a virus stock that had previously been passaged in cell culture, thus increasing the likelihood of unintentional *in vitro* mutations. In this study, we used the *in vivo* transfection technique, which allowed us to bypass the cell culture passaging steps, to more accurately characterize the development of quasispecies in pigs. Four pigs were inoculated in the lymph nodes and tonsils with RNA transcripts derived from the PRRSV infectious cDNA clone pFL12 and two pigs were inoculated similarly with PBS. Serum samples and tonsil biopsies were collected from the pigs until 63 days post inoculation

(dpi) and revealed an acute viremia that was detectable by RT-PCR at 7 and 14 dpi. During the experimental period, the remaining sera and tonsil samples tested negative for PRRSV RNA by RT-PCR. Sequencing of the ORF5 gene of 30 clones recovered from each positive serum sample identified quasispecies as early as 7 dpi, however, no mutation-rich regions were observed at these early time points. We conclude that *in vivo* transfection of PRRSV is a useful method for accurately examining quasispecies populations *in vivo* which has implications for future studies on the mechanism of PRRSV persistence and other aspects of PRRSV pathogenesis.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a disease that continues to hugely impact the global swine industry. Despite the widespread use of modified-live-vaccines and other more drastic control strategies (9, 10, 11), outbreaks are still occurring throughout the world. The causative agent of PRRS, porcine reproductive and respiratory syndrome virus (PRRSV), is heterogeneic (23) and the ORF5 gene which encodes the major envelope protein GP5 is especially variable (2, 14, 15, 16, 19, 20, 22, 24, 28). An understanding of such variability may provide some insight into the ongoing struggle against PRRS outbreaks and new possibilities for control and prevention.

Quasispecies evolution is one explanation for the heterogeneity that is observed amongst PRRSV isolates. In fact, many RNA viruses exist as quasispecies which is defined as a population of closely related yet heterogeneous sequences that are variants of one dominant sequence (5). Goldberg et al. (17) demonstrated that genetic and structural variants of PRRSV could exist between farms, among individual pigs on one farm, and

within individual pigs. They identified multiple viral variants in all of the pigs studied based on individual sample collections from naturally infected pigs. Rowland et al. (25) examined the diversity that occurs within individuals infected with PRRSV by following the mutations that develop over time in piglets infected with PRRSV while *in utero*. They determined that PRRSV does exist as quasispecies by identifying a virus subpopulation with one amino acid difference in the hypervariable ectodomain region of ORF5. Interestingly, this change also created a new glycosylation site.

PRRSV infection often results in a persistent infection (1, 4, 8, 29, 30). Little is known about the mechanism that allows the virus to survive in the host for this extended period, however, the evolution of quasispecies populations may allow for such an adaptive advantage. It is probable that the development of quasispecies population is a likely strategy for evading a neutralizing antibody response that has been generated against a specific epitope. Therefore, a better understanding of the quasispecies evolution of PRRSV may allow us further insight into the persistence of PRRSV infection as well as the failure of attempts to prevent infection with the current vaccines.

Previous studies performed to examine the quasispecies evolution of PRRSV used PRRSV that had been propagated and passaged in cell culture (6, 25). Propagation or even one passage of PRRSV in cells can result in mutations of PRRSV genome and a heterologous population in virus stock intended for quasispecies studies. These initial mutations in a non-homogeneous virus stock could not be factored into the overall evaluation of quasispecies evolution in pigs. Therefore, the cell culture propagated virus may not be suitable for *in vivo* studies intended to assess the quasispecies evolution of PRRSV in pigs. In this study, we used a recently developed infectious cDNA clone (27)

to directly infect pigs to ensure that the PRRSV originated from a homogeneous population of viral genome *in vivo* and thus allowed us to more accurately characterize all mutations that occurred over the experimental period.

MATERIALS AND METHODS

RNA Transcription. The PRRSV infectious cDNA clone pFL12 (27) was linearized by digestion with restriction enzyme *AclI*. The linearized plasmid DNA was extracted with phenol-chloroform and precipitated with ethanol. Full-length RNA transcripts of PRRSV genome were generated using the mMESSAGE mMACHINE kit (Ambion, Inc., Austin, TX) in ten 100 µl reactions each containing 50 µl 2X NTP/CAP (15mM ATP, 15mM CTP, 15mM UTP, 3mM GTP, 12mM cap analog), 10 µl 10X reaction buffer, 5 µl 30mM GTP, 6 µg (in 25 µl) of template linearized plasmid DNA, and 10 µl (200 units/µl) of T7 polymerase (18). Each reaction was incubated at 37 °C for 2 hrs. Following the incubation, the ten reaction mixtures were pooled and a 2-µl aliquot of the pooled reaction mixture was examined by agarose gel electrophoresis to check for the RNA quality and to estimate RNA concentration. The 100 µl mixture was immediately frozen on dry ice and injected into the lymph nodes and tonsils of the pigs within 3 hrs.

***In vivo* transfection of pigs with RNA transcripts of PRRSV infectious cDNA clone.** Six conventional pigs of approximately 7 weeks of age were divided into two groups. Group 1 consisted of 4 pigs (pig ID numbers 190, 191, 192, 193) and group 2 consisted of 2 pigs (pig ID numbers 177 and 178). All pigs were negative for PRRSV antibody prior to the inoculation. Each pig from group 1 was inoculated with equal amounts of the

pooled RNA transcription mixture (250 µl of the transcription mixture diluted with 350 µl of sterile, ice cold phosphate buffered saline) directly into both the superficial inguinal lymph nodes and the palatine tonsils (150 µl in each lymph node and 150 µl in each tonsil). The 2 pigs in group 2, which served as negative controls, were each mock inoculated similarly with phosphate buffered saline (PBS) in the same sites. The pigs were restrained manually for inoculation. Virginia Tech IACUC approved the animal study, and the animals were housed under conditions that met IACUC requirement.

Sample collection. Serum samples were collected prior to inoculation and weekly thereafter from each pig until 63 days post inoculation (dpi). Tonsil biopsies were collected from each pig on dpi 21, 42, and 63. For tonsil biopsies, the pigs were chemically restrained with 11mg/kg Ketamine administered intramuscularly ten minutes prior to the procedure and 2% topical lidocaine was applied to the palatine tonsils 3 minutes prior to the biopsy. The samples were collected using a dermal punch biopsy connected to a flexible rod while the mouth was retracted with a large animal mouth speculum.

ELISA. The weekly serum samples were tested by a PRRSV ELISA (IDEXX Laboratories, Inc., Westbrook, ME) at Iowa State University Veterinary Diagnostic Laboratory (data not shown). An S/P ratio ≥0.4 was considered positive for PRRSV antibodies.

RNA extraction and RT-PCR. All sera and tonsil samples were tested by RT-PCR for PRRSV RNA. Primer XHOU (5'-GCTCGAGCCATGAGGTGGGCAACCG-3') and primer HINDL (5'-GAAGCTTGGGAGCCGTGCTATCATAGC-3') were designed based on the published sequence of pFL12 parent strain 977895 (27) to amplify a 711-bp fragment beginning 66 nucleotides upstream of ORF5 start codon and ending 27 nucleotides downstream of ORF5 stop codon. The primers contained the engineered *XhoI* and *HindIII* restriction sites at the 5' and 3' ends of the amplified fragments, respectively. Total RNA was extracted with TriZol Reagent (Molecular Research Center, Cincinnati, Ohio) from 200 μl of sera or tonsil tissue homogenates. Reverse transcription was carried out at 42°C for 1 hr in a 19.5 μl reaction containing 0.5 μl RNasin, 1 μl DTT (0.1 M), 1 μl dNTPs (10mM), 0.5 μl reverse transcriptase, 4 μl 5X RT buffer, 1 μl HINDL and 11.5 μl RNA. The resulting cDNA was amplified by PCR with the following parameters: denaturation at 95°C for 9 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 2.5 min, and followed by a final incubation at 72°C for 7 min. All PCR products were examined by gel electrophoresis on a 0.8 % agarose gel.

Cloning. The PCR products from all positive samples were extracted from 0.8% agarose gel with a DNA extraction kit (Geneclean kit, Qbiogene, Morgan Irvine, CA) and eluted in DNase-free, RNase-free distilled water. The PCR products were digested with restriction enzymes *XhoI* and *HindIII* at 37°C for 2 hrs. The cloning vector pRSETA (Invitrogen, Carlsbad, CA) was also digested with *XhoI* and *HindIII* under the same

conditions. The digested PCR products and cloning vector were run on a 0.8% agarose gel and purified. For each positive PCR product, a 20 µl ligation reaction mixture containing 240 ng of the digested ORF5 insert, 80 ng of the digested pRSETA vector, 1 µl T4 DNA ligase (400 units, NE Biolabs, Beverly, MA), and 2 µl 10X T4 DNA ligase buffer was incubated overnight at 16ºC. Two microliters of the ligation reaction mixture were used to transform a 50 µl aliquot of chemically competent *E. coli* One Shot TOP10 cells (Invitrogen, Carlsbad, CA) using standard heat shock transformation techniques. Following a 1 hr. incubation at 37ºC in 250 µl S.O.C. medium, 200 µl from each culture was plated on antibiotic selective media and grown overnight at 37ºC. The following day, 30 colonies were selected from each plate, representing each positive serum sample, and grown overnight in 5 ml of Luria broth. A plasmid extraction kit (Sigma-Aldrich, St. Louis. MO) was used to extract the plasmids, which were subsequently sequenced with the PCR primers XHOU and HINDL at Virginia Bioinformatics Institute (Core Laboratory Facility, Virginia Tech, Blacksburg, VA) to determine the complete ORF5 sequence for each of the 30 individual clones.

Sequence and quasispecies population analyses. For the PCR products of each positive serum sample, the ORF5 sequences from 30 independent cDNA clones were determined. The nucleotide and amino acid sequences of the ORF5 gene regions of the 30 cDNA clones were compared and analyzed using MacVector software (MacVector, Inc., Cary, NC) to evaluate quasispecies populations.

RESULTS

Infection of pigs by *in vivo* transfection of pigs with RNA transcripts from an infectious clone of PRRSV. During the first week post-inoculation with PRRSV RNA transcripts, all of the pigs in group 1 experienced lethargy and inappetance. Following that brief period of overt illness, the pigs in group 1 exhibited no further clinical signs throughout the study. The group 2 pigs remained free of clinical signs for the entire duration of the study. Six of the serum samples from group 1 pigs that had been inoculated with the RNA transcript derived from the pFL12 infectious cDNA clone tested positive for PRRSV RNA by RT-PCR with the primers XHOU and HINDL, and the remaining samples beyond 14 dpi were negative, indicating an acute PRRSV infection. A 711-bp fragment containing the entire PRRSV ORF5 gene was amplified from the sera of pigs 190, 191, 192, and 193 at dpi 7 and pigs 191 and 192 at dpi 14. All samples from the negative control group (group 2) tested negative for PRRSV RNA by RT-PCR along with the remainder of the serum and tonsil samples from the group 1 pigs. A PRRSV ELISA confirmed that the pigs from group 1 were producing PRRSV antibodies within the first two weeks post-inoculation until the completion of the study (data not shown). The group 2 pigs did not seroconvert during the study based on a PRRSV ELISA.

Evaluation of quasispecies population. Cloning was performed on the PCR products that were amplified from all 6 positive sera samples at dpi 7 and dpi 14. Subsequently, the entire ORF5 gene sequence was determined for a total of 180 independent cDNA clones (30 clones each for six PCR-positive samples). The ORF5 sequence of the original PRRSV infectious cDNA clone pFL12 was also determined. Using MacVector

software, the nucleotide and amino acid sequences of the 30 clones from each sample at dpi 7 or 14 were aligned and compared with the original pFL12 sequence and evaluated for quasispecies development during this apparent acute PRRSV infection.

The sequences from all of the cDNA clones from dpi 7 shared 99.2 -100% nucleotide sequence identity and 98.5-100% amino acid sequence identity with each other (Table 1). Nineteen of the 120 clones (15.8%) from dpi 7 had nucleotide substitutions that resulted in amino acid changes (Fig. 1). Amongst the 60 cDNA clones at dpi 14, a 98.3-100% nucleotide sequence identity and 96.5-100% amino acid sequence identity with each other was observed. Nine of the 60 clones (15.0%) at dpi 14 had nucleotide substitutions that led to amino acid changes (Fig. 1). In general, the mutations amongst all of the cDNA clones appeared randomly distributed throughout the ORF5 gene and there were no specific regions with high mutation rates. Typically, only one clone amongst the 30 from a particular serum sample developed an amino acid mutation at any one site. No insertions or deletions were observed although two premature stop codons were created in two clones of pig 193 at dpi 7.

To more closely evaluate the evolution of PRRSV throughout the study, we configured additional alignments. The amino acid consensus sequence based on the 30 clones of each positive serum sample was determined and the aligned consensus sequences were found to be identical to each other and identical to the consensus sequence of the infectious cDNA clone pFL12. For pigs 191 and 192, alignments comparing the original pFL12 sequence with the 30 sequences from dpi 7 and the 30 sequences from dpi 14 were configured (Figs. 2, 3) and revealed no obvious substitution trends. The mutations that were present at dpi 7 within each pig typically did not recur at

dpi 14 in any of the clones within the same pig. Additionally, a comparison of all of the cloned amino acid sequences at dpi 7 to all of the cloned amino acid sequences at dpi 14 did not demonstrate any observable evolution from the original sequence of pFL12.

DISCUSSION

Studies of PRRSV quasispecies evolution *in vivo* are limited (6, 17, 25) and exclusively use cell culture-propagated and passaged virus as a starting inoculum. As the cell culture-propagated virus stock does not contain a homogeneous population of virus, the nature of quasispecies evolution, especially during acute infection in pigs, may not reflect reality. In this study, we used PRRSV *in vivo* transfection (21) to more accurately examine the nature of PRRSV quasispecies in young pigs during an acute infection. The use of the PRRSV infectious cDNA clone as starting material ensured that the original population of PRRSV was homogeneous, thereby avoiding any mutations that likely would have occurred during cell culture propagation or passaging. We showed that the PRRSV infection resulting from direct inoculation of infectious cDNA clone-derived RNA transcripts into the tonsils and lymph nodes is similar to a natural infection and allows a novel approach for evaluating quasispecies evolution *in vivo* without the concern of undesirable mutations resulting from *in vitro* virus propagation or passaging prior to inoculation.

The inoculated pigs appeared to develop an acute infection, and thus our analyses of quasispecies population were limited to a two week infection period (dpi 7 and dpi 14). Based on the analyses of 30 individual cDNA clones from each of the 6 PCR-positive pigs at dpi 7 and dpi 14, quasispecies populations were identified in each PCR-positive

pig (Fig. 1, Table 1). A 1.5% variation was detected amongst the amino acid sequences of the clones at dpi 7, while up to 3.5% amino acid sequence variation was observed at dpi 14. Although the PRRSV mutation rate appears to have increased during the second week of replication, this change in variation may be explained by one serum sample containing one aberrant clone that contained nine nucleic acid substitutions (Fig. 1) and an increased number of clones with 3 amino acid substitutions. Due to the small sample size (ie. the number of positive serum samples at dpi 14), these results are likely not significant. In general, the PRRSV quasispecies sequences lacked obvious hot spots of mutations that might indicate an evolutionary trend of PRRSV as it mutates within an animal.

Multiple sequence alignments of the 30 clones from each serum sample revealed an absence of recognizable evolution and a greater tendency towards stabilizing selection during the first few weeks of PRRSV replication. The amino acid consensus sequences from dpi 7 and dpi 14 pigs were found to be identical to the sequence of the infectious cDNA clone pFL12. In addition, no obvious mutation trends were demonstrated over time in two pigs, pig 191 and pig 192, whose serum was positive for 2 weeks post-inoculation (Figs. 2,3). The randomness of the mutations and the absence of amino acid changes in the consensus sequences indicate a lack of any signs of adaptation that might be revealed during the later stages of viral replication.

PCR may generate random mutations that could be mistaken for quasispecies in the clones. The reported substitution rate using *Taq* polymerase is approximately 1/10,000 to 1/100,000 mutations based on the reaction conditions (12, 26). This calculation translates into approximately 1 mutation per 16 clones. The substitutions in

this study clearly surpass this rate demonstrating that the observed mutations are not simply random changes caused by PCR amplification.

A more prolonged or persistent infection would have provided more time for mutations to arise. In previous quasispecies studies, obvious sites of mutations have been observed in GP5 as PRRSV replicates. Rowland et al. (25) identified a population of Asn-34 mutants that created a new glycosylation site in GP5. Based on recent reports with PRRSV and similar viruses, the glycosylation pattern in an epitope region can have an effect on cell tropism, infectivity, immunogenicity, and neutralizing antibody interaction (3, 7, 13). In our study, the acute infection limited such observations and may have been caused by many factors including the nature of the acute strain of PRRSV that was used to develop the infectious cDNA clone and the age of the pigs at the time of inoculation. Additionally, we recognize that nested RT-PCR might have detected lower levels of virus in the serum and tissues at a later time point, however, nested RT-PCR would also have increased the likelihood of introducing additional artificial PCR mutations during amplification. Therefore, we were unable to logically prolong our detection of the infection period without sacrificing the reliability of our results.

In summary, we have demonstrated that *in vivo* transfection is a desirable method for examining quasispecies without the concern of unwanted mutations resulting from virus propagation or passaging procedures prior to inoculation of animals. We were able to document that a quasispecies population can develop as early as 7 days after infection when pigs were inoculated with a homogeneous population of PRRSV. We hypothesize that this quasispecies evolution would continue throughout a persistent infection, ultimately demonstrating adaptations that could increase viral fitness.

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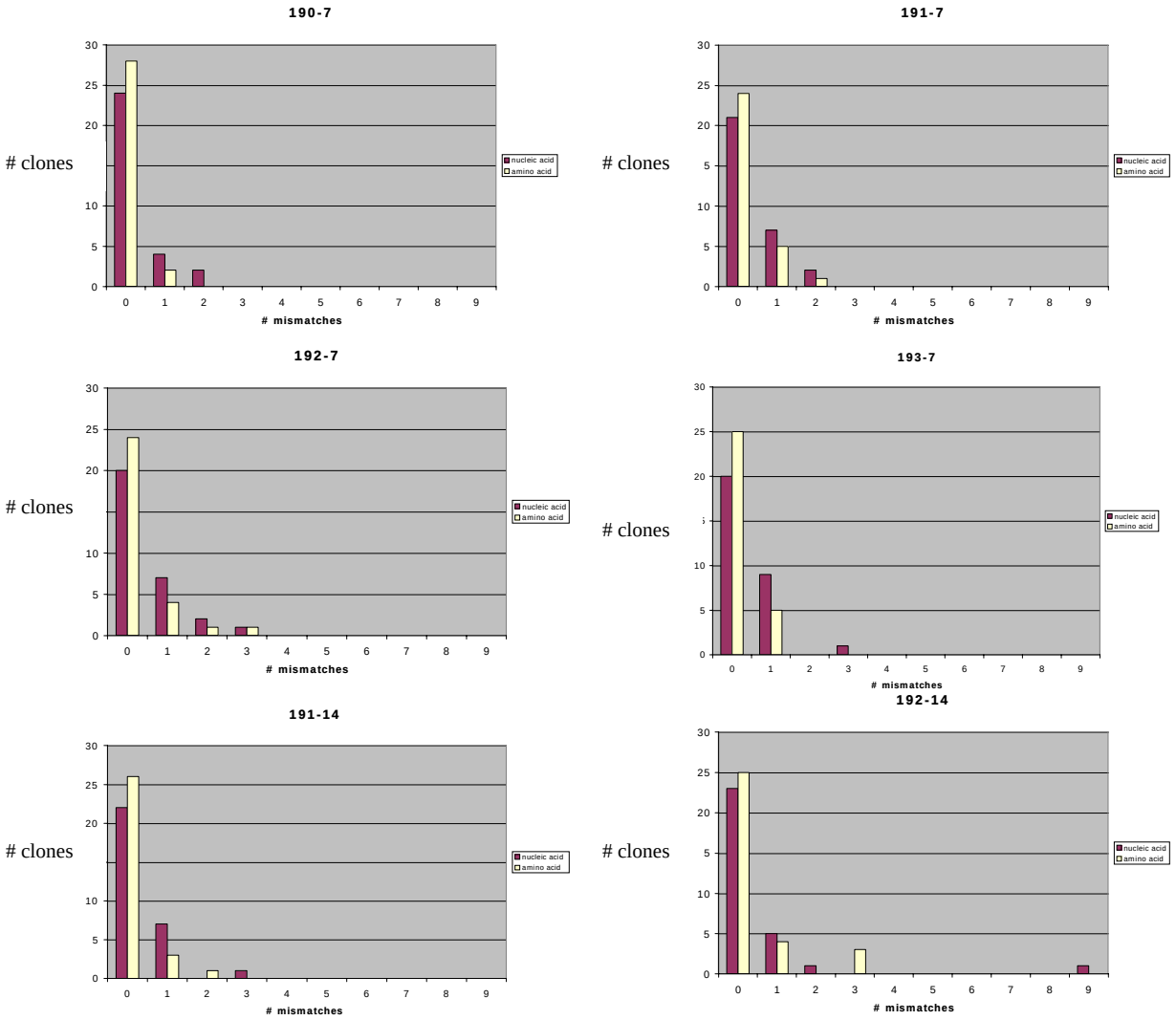

Fig. 1

Figure 5.1. Number of nucleic acid and amino acid substitutions observed amongst 30 clones isolated from each of 6 PCR-positive serum sample at dpi 7 and dpi 14. Y-axis indicates the number of cDNA clones of the 30 that were sequenced that had the indicated number of mutations, and the X-axis indicates the number of mutations that were present in the ORF5 region when compared to the ORF5 of the inoculated strain (infectious cDNA clone pFL12.)

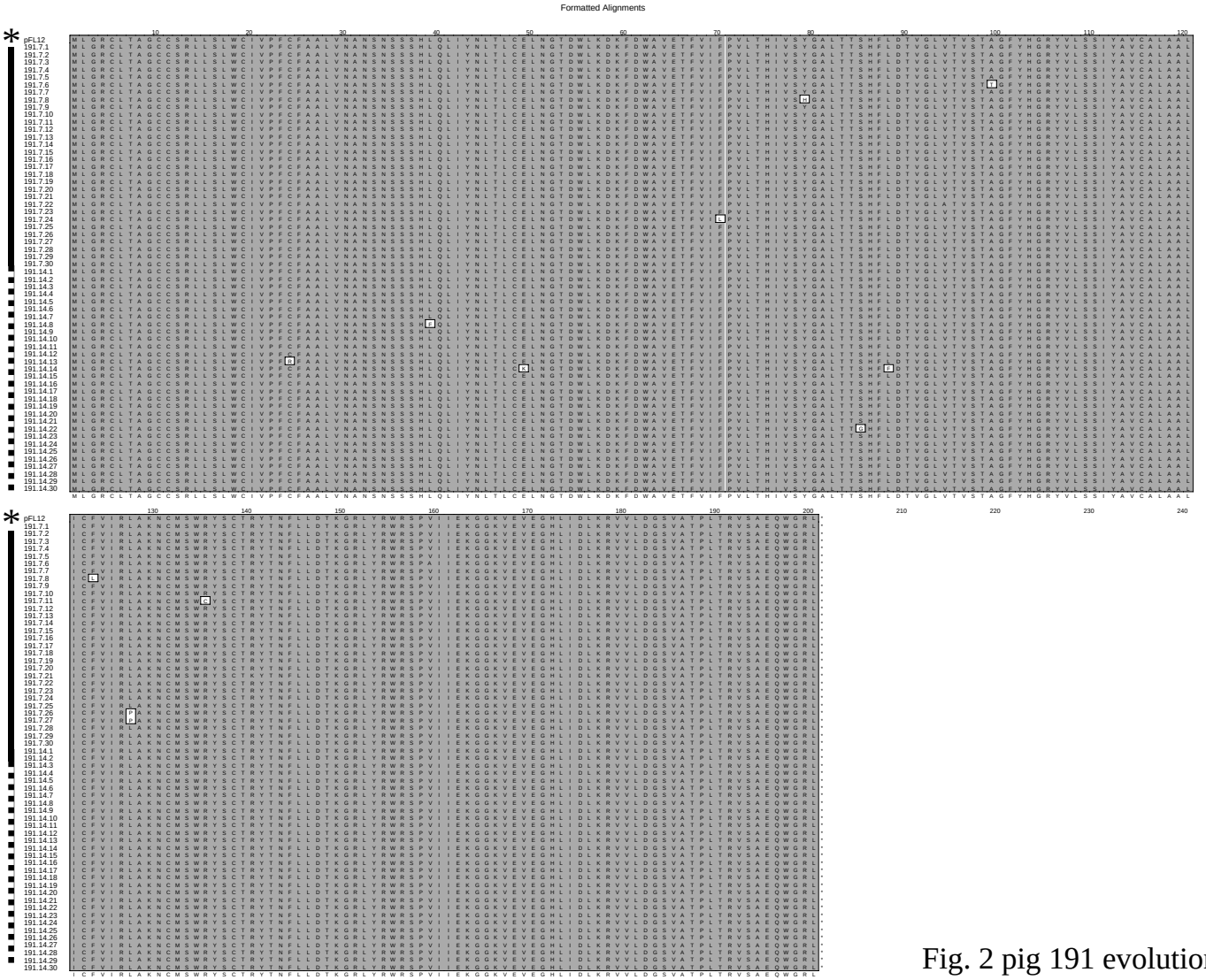

Fig. 2 pig 191 evolution

Figure 5.2. GP5 evolution in pig 191. Amino acid sequence alignment of GP5 protein sequence of infectious cDNA clone pFL12 (asterisk), 30 clones sequenced from pig 191 at dpi 7 (solid bar), and 30 clones sequenced from pig 191 at dpi 14 (dashed bar.)

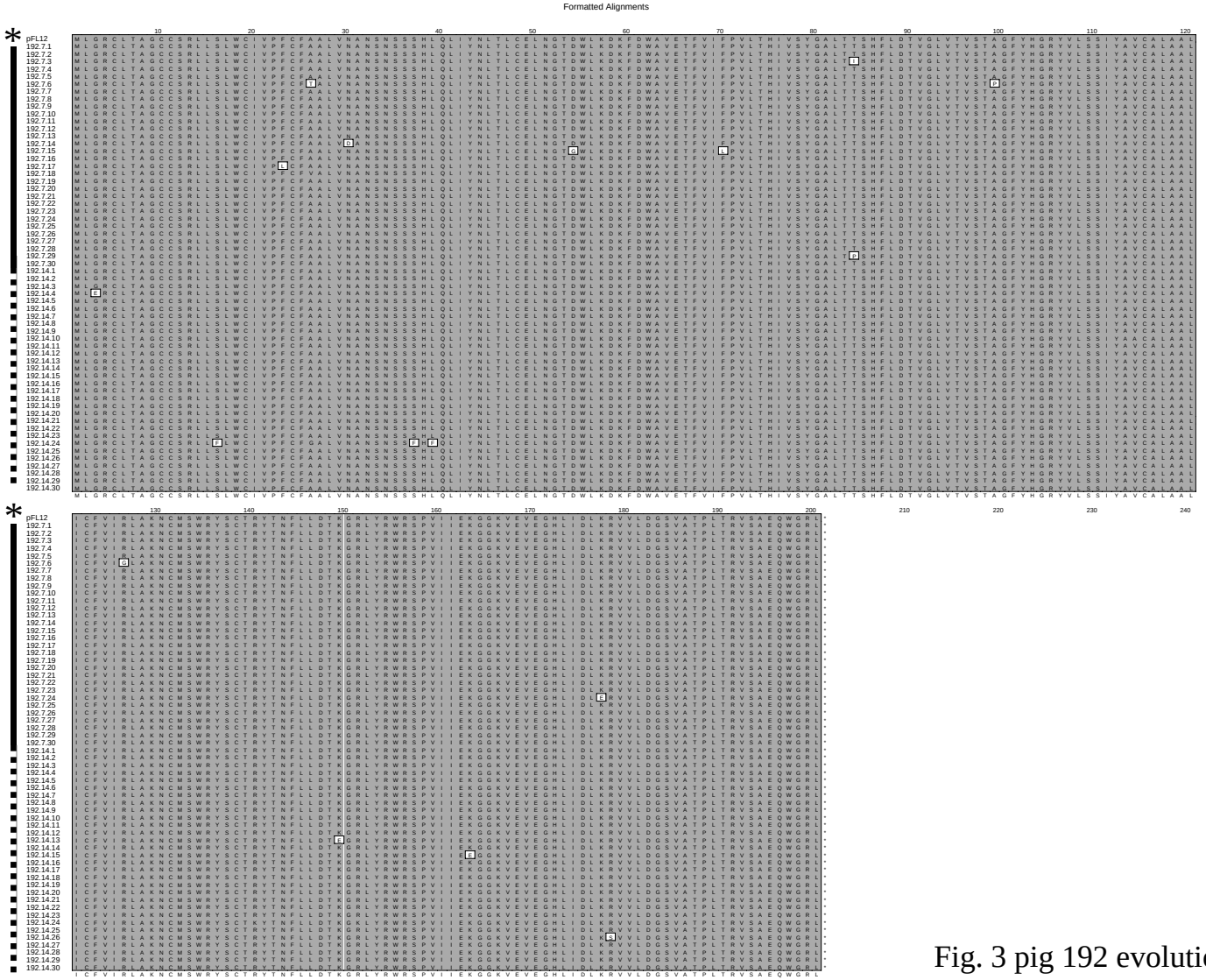

Fig. 3 pig 192 evolution

Figure 5.3. GP5 evolution in pig 192. Amino acid sequence alignment of GP5 protein sequence of infectious cDNA clone pFL12 (asterisk), 30 clones sequenced from pig 192 at dpi 7 (solid bar), and 30 clones sequenced from pig 192 at dpi 14 (dashed bar.)

Table 5.1. Sequence variation and quasispecies during an acute PRRSV infection in pigs inoculated with a homogeneous population of PRRSV derived from an infectious cDNA clone

	DPI	NT seq identity range (%)*	AA seq identity range (%)
Pig ID #			
190-7	7	99.3/100	99.0/100
191-7	7	99.3/100	98.5/100
192-7	7	99.2/100	98.5/100
193-7	7	99.3/100	99.0/100
191-14	14	99.3/100	98.5/100
192-14	14	98.3/100	96.5/100

*Values are expressed as minimum/maximum identity upon comparison with the original sequence from infectious cDNA clone pFL12. For each sample, 30 individual cDNA clones were sequenced and compared.

Chapter 6

Expression of PRRSV GP5 envelope protein in attenuated *Salmonella choleraesuis* and evaluation of its use as an oral vaccine

K.F. Key, M. Seleem, J. DiCristina, N. Sriranganathan, S.M. Boyle, and X.J. Meng

ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) continues to devastate the global swine industry despite the widespread use of modified live vaccines (MLVs) and other control strategies. A recent study indicates that PRRS is responsible for approximately half a billion dollars in losses annually in growing pig populations and breeding herds in the U.S. Evidence of vaccine failure and reversion to pathogenic phenotypes clearly demonstrates the need for a newer generation of PRRS vaccines. *Salmonella choleraesuis* is another major swine pathogen that has recently been used, in its attenuated form, to deliver foreign antigens that provide protection against major diseases. In this study, we evaluated the ability of *S. choleraesuis* to express the PRRSV major envelope protein, GP5, *in vitro* and tested its immunogenicity in mice. The PRRSV ORF5 gene, which encodes GP5, or a portion of the gene minus its signal sequence was cloned into a prokaryotic expression vector to generate two recombinants (rec1 and rec2), respectively. Additionally, the luciferase (Lux) gene and the green fluorescent protein (GFP) gene were each cloned into the same prokaryotic vector to generate two additional recombinants (rec3 and rec4). Expression of a ~23 kDa protein,

which is similar to the predicted size of PRRSV GP5 protein, by *S. choleraesuis* was detected by anti-His serum and a GP5 monoclonal antibody PP5dB4 in western blot analyses. However, N-terminal sequencing and western blot analyses with other PRRSV antibodies could not definitively confirm the expression of GP5. A pilot immunogenicity study was subsequently conducted to determine if recombinants could induce GP5 antibodies in mice. Seven groups of six mice each were administered with oral and intraperitoneal doses of the *S. choleraesuis* recombinants (recs 1-4) along with one group that received *S. choleraesuis* without the vector as a negative control and one group that was administered semi-purified PRRSV virion proteins intramuscularly as a positive control. The mice were boosted twice with their respective inocula. Although the positive control mice generated PRRSV-specific antibodies, an indirect immunofluorescence antibody test of sera and culture of fecal samples revealed no indication of *Salmonella* replication in the other mice or any evidence of antibody production against *S. choleraesuis* or PRRSV GP5. Despite the inability to construct an effective *Salmonella*/PRRSV vaccine, this study has implications for the planning and analysis of future vaccine studies in this area.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) outbreaks remain a concern in the swine industry despite the widespread use of modified live vaccines (MLVs), which decrease the clinical signs of PRRSV. PRRSV is genetically heterogenic (11,19, 23, 27, 28, 29). The European and North American strains share only a 70% nucleotide sequence identity and represent two separate genotypes (21). Significant

genetic variation also exists amongst the North American isolates (11, 22, 23). Therefore, the current vaccines, which are all based on a single PRRSV strain, are unlikely to provide complete protection against a group of viruses with such variation in their genome. Additionally, evidence exists supporting the reversion of the MLV RespPRRS to a virulent phenotype (18, 24, 25). Madsen et al. (18) demonstrated that several Danish strains of PRRSV originated from the MLV RespPRRS. As further indication that the current vaccines are not completely effective, the outbreaks of "acute" PRRS began to surface in late 90s in swine herds that had previously been vaccinated with MLVs. The outbreaks were characterized by increased numbers of abortions and stillborn piglets and higher preweaning mortality (3). Based on these various findings, a safer and more effective PRRS vaccine is clearly in great demand.

Protection against PRRS is most likely provided by both humoral and cell mediated immunity. Presently, there is more research supporting the importance of the humoral immune response. Multiple reports indicate that neutralizing antibodies prevent or block viremia (31, 48). ORF5 contains the major neutralization epitope. Pirzadeh and Dea (34) demonstrated that vaccination of pigs with plasmid DNA encoding PRRSV ORF5 produced a decrease in macroscopic lung lesions and in generalized viremia to challenged pigs. Other reports have also indicated that immunization with envelope protein GP5 is protective (2, 33, 34).

In addition to PRRS, porcine salmonellosis is another devastating swine disease. *Salmonella choleraesuis* is a facultative intracellular pathogen that is responsible for the majority of salmonellosis outbreaks in swine. The pathogen causes paratyphoid in swine and is typically transmitted via the fecal-oral route (46). According to the National

Animal Health Monitoring Service, approximately 100 million dollars are lost in the U.S. swine industry annually due to porcine salmonellosis (42). Attenuated *Salmonella* vaccines have been developed and have been shown to be both stable and effective in protecting pigs challenged with a virulent *Salmonella* strain (14, 37, 38). The vaccines are believed to stimulate secretory, humoral, and cell-mediated immunity (20).

Recently, attenuated *Salmonella* vaccine strains have been used as delivery vehicles for various bacterial and viral antigens including those of *Escherichia coli* (1), Mycoplasma (7), HIV (8), herpes simplex virus (12), and hepatitis B virus (40). Because a *Salmonella* vaccine usually enters into the gut-associated lymphoid tissue, these systems allow *Salmonella* to deliver the antigen to the host's immune system. The expression of a foreign antigen by *Salmonella* potentiates the generation of antigen-specific secretory, humoral, and cell-mediated immunity. Therefore, the development of a vaccine that utilizes attenuated *Salmonella* as a delivery vehicle for GP5 would be ideal for providing protection against these two major diseases that continue to plague the swine industry.

In the current study, we made an effort to evaluate the feasibility of a *Salmonella choleraesuis* vaccine strain expressing the GP5 protein of PRRSV and its ability to induce a specific immune response in mice. We hypothesized that the dual vaccine would be immunogenic in mice, triggering an immune response to both *S. choleraesuis* and PRRSV, and ultimately leading to an effective PRRS vaccine.

MATERIALS AND METHODS

Animals, bacterial strains, virus isolate. Forty-two six-week-old female BALB/c mice were purchased and housed three mice per cage. One additional mouse was housed separately. The animal study was approved by Virginia Tech IACUC and the animals were housed under conditions that met IACUC requirement. The commercial attenuated *Salmonella choleraesuis* vaccine strain (Enterisol SC-54) was provided by Dr. Eric Vaughn (Boehringer-Ingelheim Vetmedica Inc.) and was reconstituted as indicated. Throughout the study, the bacteria were grown in tryptic soy broth (TSB) and on TSB agar (TSA) at 37ºC. *Salmonella* cultures containing the plasmid with the chloramphenicol resistance gene were grown in TSB with chloramphenicol (30 µg/ml). The PRRSV ORF5 cDNA used for the recombinant vector construction was amplified from the PRRSV isolate ATCC VR2385 (ISU-12), a North American strain of the virus. Semi-purified virion was generated for inoculation of mice that were positive controls in the immunogenicity study by clarification of 150 mls of pooled PRRSV-infected cell culture stocks at 9000 rpm for 20 min at 4ºC followed by ultracentrifugation of the supernatant at 28,000 rpm for 3 hrs at 4ºC on a 30% sucrose cushion. The virus pellet was redissolved in 600 µl of TBS (pH 7.4). The presence of PRRSV proteins was confirmed by western blot analysis using porcine convalescent sera and a PRRSV GP5 monoclonal antibody.

Construction of expression vectors. PRRSV RNA was extracted from ISU-12 infected MARC-145 cells (Dr. K.J. Yoon, Iowa State University College of Veterinary Medicine) and RT-PCR was performed to amplify the entire PRRSV ORF5 region (603 bp) as well

as a portion of the ORF5 gene minus the 32 amino acid signal sequence at the 5' end. After testing various promoters and vectors to optimize the expression of the PRRSV protein, we opted to use a pNS prokaryotic expression vector with a Trc promoter and a chloramphenicol resistance gene as the backbone for the construct (Seleem et al., to be submitted for publication). All of the constructs contained a 6x His tag at the 5' end of the insert. Numerous recombinants were constructed for this study using two unique restriction sites to facilitate directional cloning. Recombinant 1 (rec1, pNSTrcSSWildSS) was constructed by cloning a *Salmonella* signal sequence and the entire PRRSV ORF5 gene into the expression vector. Recombinant 2 (rec2, pNSTrcCh2HisWild) was constructed by cloning the PRRSV ORF5 gene minus the first 32 amino acids that encode its signal sequence into the expression vector. Recombinant 3 (rec3, pNSTrcLux) and recombinant 4 (rec4, pNSTrcDGFP) were constructed by cloning the luciferase (Lux) gene of *Photorhabdus luminescens,* a bioluminescent bacteria, and the green fluorescent protein (GFP) gene of *Aequorea victoria* into the expression vector, respectively. The inserts of each recombinant vector containing PRRSV ORF5 were confirmed by RT-PCR and sequencing (Virginia Bioinformatics Institute, Virginia Tech., Blacksburg, VA) prior to expression analysis. The expression of the Lux gene was confirmed by examination of culture plates containing rec3 *S. choleraesuis* colonies for bioluminescence while the expression of the GFP gene was confirmed by observation of the rec4 *S. choleraesuis* colonies under ultraviolet light.

Western blot analysis of GP5 expression. *S. choleraesuis* containing the various recombinant vectors were grown up overnight in 20 mls of TSB. The bacteria were

harvested by centrifugation and resuspended in 1 ml guanidine-HCl and gently mixed for 2 hrs. The suspension was pelleted at 10,000g for 15 min. The supernatant was collected and the His-tagged proteins were purified using the Ni-NTA Spin Kit (Qiagen, Valencia, CA) and eluted in buffer. To remove the guanidine-HCl from the protein samples, dialysis was performed with Microcon (Millipore Corp., Bedford, MA) centrifugal filters for 1.5 hrs. Western blot analysis was performed by loading the purified proteins on a 12% Bis-Tris reducing gel (Invitrogen, Carlsbad, CA). The proteins were transferred to a nitrocellulose membrane that was incubated overnight with anti-His serum (1:5000). Additional membranes that were prepared similarly were incubated with PRRSV GP5 monospecific antibody (1:400), monoclonal antibody PP5dB4 (1:50), or PRRSV polyclonal antiserum (1:500) followed by incubation with anti-rabbit-HRP (1:1000), anti-mouse-HRP (1:4000) and anti-swine-HRP (1:1000) sera, respectively. The membranes were developed using a colorimetric method. Some of the identified proteins were further analyzed by N-terminus sequencing at Johns Hopkins University Sequencing Facility (Baltimore, MD).

Mice immunization. Forty-two female BALB/c mice were initially divided into seven groups of six mice each. On the day of inoculation, feed was withheld from the mice. *Salmonella* was delivered orally using sterile 38.1 mm, 1.5" gavage needles. Refer to Table 1 for the specific inoculum and the inoculation scheduling of each group. Initially, the *Salmonella choleraesuis* and the *S. choleraesuis* recombinants were administered $1x10^6$ CFU in 10% sodium bicarbonate buffer. The positive control group received 40 µg of PRRSV semi-purified virion (prepared 1:1 with Freund's incomplete adjuvant

(Sigma-Aldrich, St. Louis, MO)) intramuscularly (IM) for the first inoculation. The adjuvant was not included in the additional boosters. One week later, half of each group was boosted orally with $1x10^6$ CFU of *S. choleraesuis* while half of each group was boosted intraperitoneally (IP) with $1x10^4$ CFU of *S. choleraesuis*. Because there were three mice per cage, and two cages per group, there was no intermingling between the mice with different inoculation methods. At 3 weeks post-inoculation, our intention was to boost the mice a second time with $1x10^8$ CFU of *S. choleraesuis* orally and $1x10^6$ CFU of *S. choleraesuis* administered IP. When this IP dose proved to be fatal to the first few mice after only a few minutes post-inoculation, the remaining mice were inoculated orally with $1x10^8$ CFU. One of the rec1 groups was boosted with the semi-purified PRRSV rather than the *Salmonella* recombinant. (Table1)

Sample collection. Feces was collected from the mice cages weekly and blood was drawn from the orbital sinus of each mouse every two weeks from 0 days post-inoculation (dpi) until 70 dpi. The mice were anesthetized with isoflurane prior to the blood collection. The serum was isolated and stored at -80ºC until IFA testing was performed.

Bioluminescence assay. On the day of the inoculation, the mice from group 7 that had received the *S. choleraesuis* strain expressing the Lux gene were examined for bioluminescence using an IVIS imaging camera (Virginia Tech, Blacksburg, VA) in order to monitor *Salmonella* replication in these mice. One mouse from the group was

again examined every one to two days post-inoculation for the following week. This procedure was repeated for this group of mice after each booster inoculation.

Bacterial isolation. A 10% fecal solution was prepared in phosphate buffered saline (PBS) (pH 7.4). A 10-µl aliquot of the fecal slurry was inoculated into 2 mls of TSB and incubated at 37ºC. After a three hour incubation, the culture was plated on TSB and chloramphenicol plates as well as XLT4 agar plates (PML Microbiologicals, Wilsonville, OR) and incubated overnight. A 100-µl aliquot of the slurry was also directly plated on TSB and chloramphenicol and XLT4 plates and treated similarly.

***Salmonella* agglutination test.** To test for *Salmonella* antibodies in the mouse sera, ten microliters of mouse sera was combined with one *S. choleraesuis* colony and manually rotated for one minute on a sterile glass plate. Agglutination levels were examined and recorded. A positive control was performed by combining 10 µl of *Salmonella* O antiserum (Difco, Detroit, MI) with a *S. choleraesuis* colony.

Indirect immunofluorescence assay (IFA). Due to the absence of a standardized ELISA for the detection of GP5 PRRSV antibodies in mouse sera, IFA was performed on each of the serum samples. Eight-well Labtek chambers that were 90-100 % confluent with MARC-145 cells were each infected with 100 µl of PRRSV isolate ISU-12 and incubated for 48-72 hrs. Following the incubation period, the cells were washed with 200 µl PBS and fixed with 100 µl cold 80% acetone and 20% methanol solution at 4ºC for 15 min. After another PBS wash, a 100-µl aliquot of a 1:10 dilution of the mouse sera was

added to each well and incubated at 37ºC for 1 hr. The cells were washed three times with PBS and 100 µl of a 1:50 dilution of FITC-labeled goat anti-mouse IgG (KPL, Gaithersburg, MD) was added to each well. The chambers were again incubated at 37ºC for 45 min. and then washed three times with 200 µl PBS. All of the slides were evaluated for a fluorescent signal under a fluorescence microscope.

RESULTS

GP5 expression in *S. choleraesuis*. Western blot analysis with the purified proteins collected from the recombinant *S. choleraesuis* identified a protein that reacted with both the anti-His serum and a GP5 monoclonal antibody, PP5dB4; the band migrated at approximately 23 kDa, which is the expected size of PRRSV GP5 (Fig. 1). However, N-terminus sequencing (Johns Hopkins University, Baltimore, MD) of the abundant protein identified a chloramphenicol acetyl transferase (CAT) protein also expressed by the prokaryotic expression vector. It was not possible to definitively identify PRRSV GP5 by protein sequencing. However, the likelihood of its expression by *S. choleraesuis* was not excluded since the GP5 protein and the abundant CAT protein are a similar size and thus were not resolved in the denaturing gel.

Clinical observations. Following the initial mouse inoculation, all of the mice in the *Salmonella*-inoculated groups appeared unthrifty and remained in that condition throughout the study. The mice inoculated IP exhibited a higher level of lethargy and depression than the orally inoculated mice. Two of the mice developed necrotic tissue in the caudal abdominal area from the *Salmonella* inoculations. Seven mice died

prematurely apparently due to both toxicity and anesthetic/experimental stress. The positive control group inoculated IM with purified PRRSV proteins had no signs of clinical disease.

***Salmonella* isolation, antibody responses, and luciferase expression *in vivo*.** A number of tests were performed to document the replication of the *S. choleraesuis* strains in the inoculated mice. After plating of the fecal cultures, no colony growth was observed on either the TSB and chloramphenicol plates or the XLT4 plates demonstrating a lack of fecal shedding of the *S. choleraesuis* recombinants in the mice. Additionally, agglutination tests to examine whether the mice were generating *Salmonella* antibodies were negative. However, the positive control anti-*Salmonella*-O sera did cause agglutination when mixed with a *S. choleraesuis* colony. The group 7 mice that were inoculated with *S. choleraesuis* expressing the Lux gene (rec3) did not display any bioluminescence except for a short period immediately following intraperitoneal inoculation with the first booster (Fig. 2). These mice were negative for bioluminescence by the following day. The plated rec3 *S. choleraesuis* colonies did exhibit luminescence when examined, thereby demonstrating that the Lux gene was being expressed by the bacteria prior to inoculation.

Antibody responses of mice. To test for antibody production against PRRSV GP5 expressed by *S. choleraesuis*, seven groups of mice were inoculated and boosted both orally and IP with various *S. choleraesuis* recombinants containing a PRRSV ORF5 gene insert. Serum was collected every two weeks and was tested by IFA for the presence of

GP5 PRRSV antibodies. By IFA analysis, the serum remained negative for PRRSV GP5 antibodies throughout the study. The positive control group, which had been inoculated IM with semi-purified PRRSV virion proteins, produced GP5 PRRSV antibodies that were clearly visible by IFA (Fig. 3).

DISCUSSION

In this study, we evaluated the feasibility of *S. choleraesuis* to express the PRRSV major envelope protein, GP5, as well as the immunogenicity of the recombinant *S. choleraesuis* in mice. The expression of a fusion PRRSV GP5 recombinant protein by *Salmonella* was detected by anti-His serum that is specific for the 6x His-tag sequence engineered to the viral protein. However, further western blot analyses with PRRSV antibodies could not definitively confirm the GP5 expression due to the nonspecific binding to the CAT protein that is approximately the same size as the PRRSV GP5 protein. N-terminus sequencing revealed that the predominant sequence at the expected site of PRRSV GP5 was CAT. Because protein sequencing will only identify the dominant sequence, we speculate that the GP5 protein is being masked by the abundant CAT protein. The possibility that *S. choleraesuis* failed to express GP5 is not likely considering sequencing of the constructs which confirmed the gene's orientation and the reading frame with respect to the promoter. Moreover, the fusion protein was isolated using a His-binding column and reacted with 6x His monoclonal antibodies. Additionally, *S. choleraesuis* and other *Salmonella spp.* have routinely and successfully been used to express foreign antigens (1, 4, 5, 6, 7, 8, 9, 10, 12, 13, 16, 17, 30, 32, 35, 36, 39, 40, 43, 47, 40).

A pilot mouse study was performed despite our inability to definitively confirm the GP5 expression in *Salmonella*. Following the mouse study, we concluded that the *S. choleraesuis* strains were not replicating within the inoculated mice based on our failure to reisolate *Salmonella* in the mouse feces and the inability to find specific *Salmonella* antibodies. The fact that the negative control mice inoculated with nonengineered *S. choleraesuis* also failed to develop a *Salmonella* infection indicates that the strain was not capable of producing a productive infection. This in direct contrast to previous studies showing that BALB/c mice inoculated orally with *S. choleraesuis* expressing a foreign protein can generate *Salmonella* antibodies in the serum as well as antibodies to the foreign protein (36,43,44,45). According to Stabel et al. (45), peak colonization of *S. choleraesuis* in abdominal tissues of mice can be found around 7 dpi. In explanation, the *S. choleraesuis* vaccine strain, intended for pigs, was not adapted to mice prior to the vaccine study which most likely contributed to its failure to replicate. Besides adaptation, the main difference between our study and previous studies was a lower initial starting dose. However, studies have also shown that mice can develop antibodies when receiving oral doses of *S. choleraesuis* as low as $1.9x10^5$ CFU (43). In our case, the lower starting dose ($1x10^6$) was unintentional and resulted from a 2-log decrease in bacteria titer during a storage period. Our assays for evaluating *Salmonella* infection and GP5 antibodies were also not ideal and standardized ELISAs would have provided more sensitive and accurate methods for analysis. Naturally, the lack of *Salmonella* replication prevented the expression of GP5 at detectable levels, thus inhibiting us from further evaluating the efficacy of our constructs.

A *Salmonella*/PRRSV combined vaccine would provide multiple advantages over other vaccine options that are currently used for PRRS prophylaxis. Recent studies indicate that protection resulting from PRRSV vaccination is generally strain-specific (41, 55). With the presence of such a heterogenic population, a multivalent DNA vaccine delivered by *S. choleraesuis* may allow for the utilization of the genes of multiple PRRSV isolates to generate one universally protective vaccine. Another advantage of a DNA vaccine deliverd by *S. choleraesuis* is the ability to orally administer the vaccine compared to DNA vaccines that are typically delivered by intramuscular or intradermal injection. The live recombinant vaccine may also be less expensive to produce compared to a highly purified DNA vaccine (20). These benefits, plus the likelihood of generating immunity against two major pathogens, suggest that such a vaccine could greatly improve PRRSV management and control. While this pilot study did not generate a new recombinant vaccine, it has provided a wealth of information about the hurdles faced with a *Salmonella/*PRRSV construct and the evaluation of its immunogenicity; this includes dose, infectivity, and level of antigen expression. This information will aid in future studies to further develop this promising vaccine.

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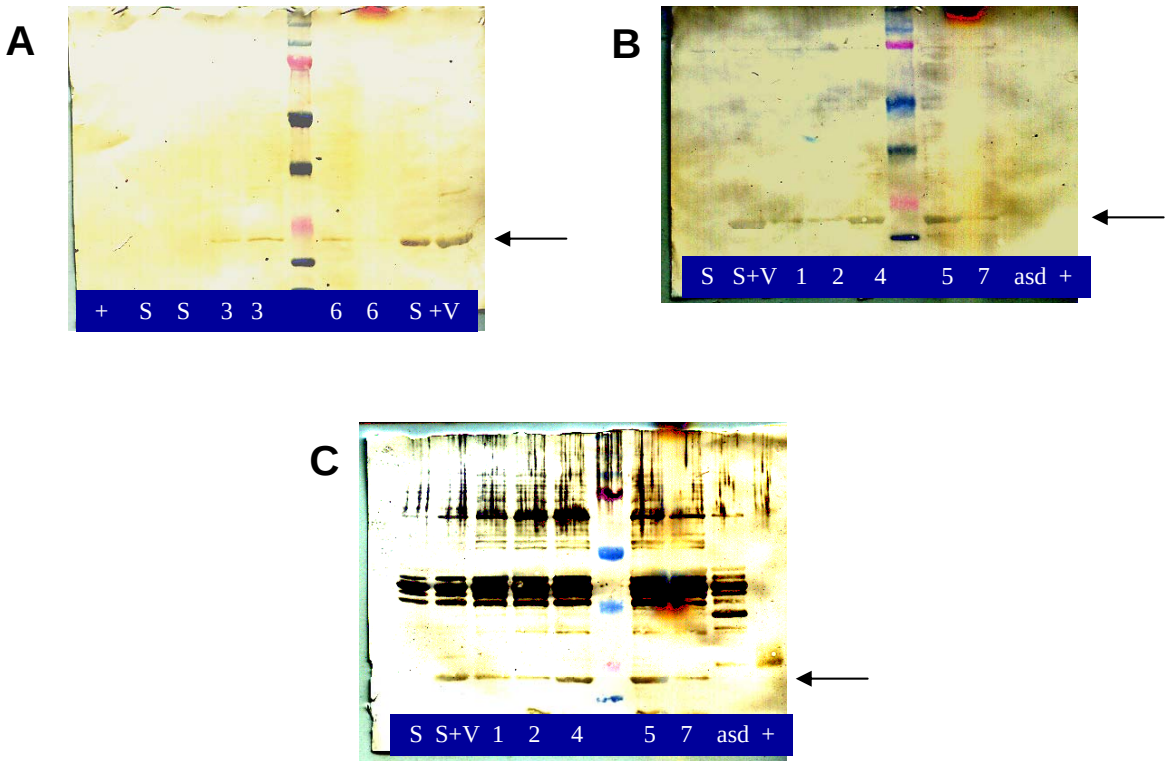

Figure 6.1. Western blot analysis of PRRSV GP5 expression in *Salmonella*. Western blot membranes are pictured in which *Salmonella choleraesuis* samples containing a PRRSV ORF5 gene insert were evaluated for GP5 expression. Panels A and B indicate membranes in which PRRSV monoclonal antibody PP5dB4 was reacted with the membranes followed by HRP-conjugated anti-mouse. In panle C, a PRRSV monospecific antibody generated against ORF5 was used as the primary antibody followed by HRP-conjugated anti-rabbit. **S:** *S. choleraesuis* (negative control); **S+V:** *S. choleraesuis* + vector (negative control); **1-7:** various recombinant vector constructs containing ORF5 insert; **asd:** asd vector construct containing ORF5 insert; **+:** PRRSV purified virion. The arrows in each panel indicate the 23 kDa protein bands.

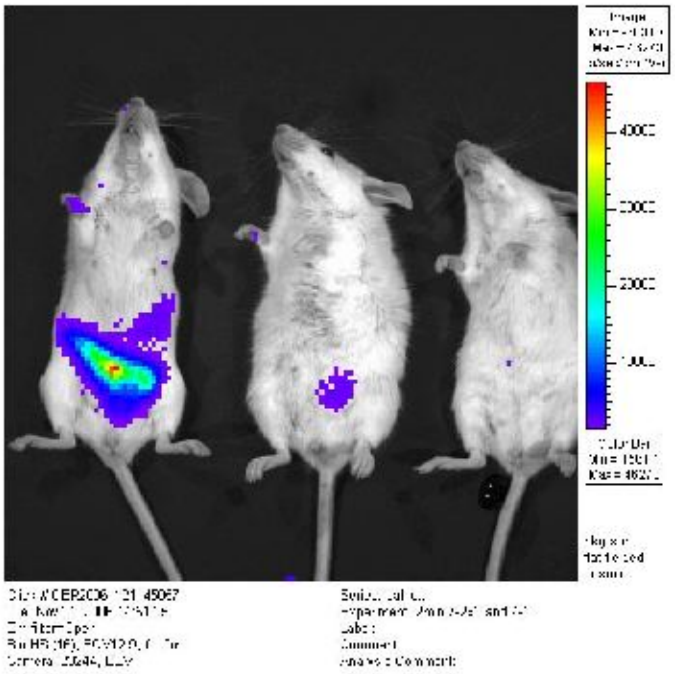

Figure 6.2. Bioluminescence observed in *S. choleraesuis* rec 3 group a few hrs. post-inoculation with the first booster dose of *S. choleraesuis* screening constructs expressing the luciferase gene. The two mice on the left were inoculated intraperitoneally (1 x 10^4 CFU *S. choleraesuis* rec3) and the mouse on the right was inoculated orally (1 x 10^6 CFU *S. choleraesuis* rec3).

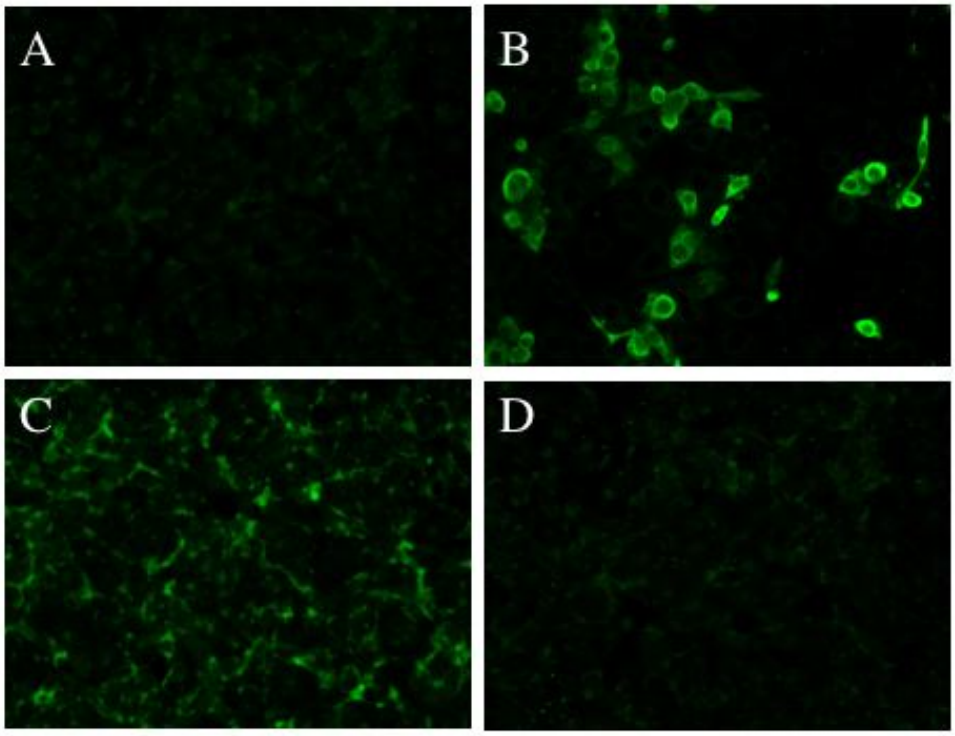

Figure 6.3. IFA of MARC-145 cells infected with PRRSV to detect anti-GP5 PRRSV antibodies in mouse serum. All cells were infected with PRRSV ISU-12, and were stained with the mouse sera from the study followed by FITC conjugated anti-mouse IgG. These results were from 28 dpi although similar results were observed throughout the study. The primary antibodies for each panel were (A) Negative control, serum from mouse inoculated with *S. choleraesuis*. (B) Positive control, PRRSV monoclonal anti-nucleocapsid antibody SDOW17. (C) Positive control, serum from mouse inoculated with semi-purified PRRSV virion proteins. (D) serum from mouse inoculated with rec2. All other recombinants demonstrated similar results as those seen in panel D.

Table 6.1. Inoculation schedule of mice receiving *S. choleraesuis* or a *S. choleraesuis* recombinant containing PRRSV ORF5

				Inoculum and route		
Group #	**Group identity**	**construct**	**# mice**	**dpi 0**	**dpi 7**	**dpi 21**
1a	Rec1	SC+ pNSTrcSSWildSS	3	$1X10^6$ rec1, PO	$1X10^6$ rec1, PO	$1X10^8$ rec1, PO
1b	Rec1	SC+ pNSTrcSSWildSS	3	$1X10^6$ rec1, PO	$1X10^4$ rec1, IP	$1X10^6$ rec1, IP
2a	Rec1	SC+ pNSTrcSSWildSS	3	$1X10^6$ rec1, PO	$1X10^6$ rec1, PO	40 µg PRRSV, IM
2b	Rec1	SC+ pNSTrcSSWildSS	3	$1X10^6$ rec1, PO	$1X10^4$ rec1, IP	40 µg PRRSV, IM
3a	Rec2	SC+ pNSCh2HisWild	3	$1X10^6$ rec2, PO	$1X10^6$ rec2 PO	$1X10^8$ rec2, PO
3b	Rec2	SC+ pNSCh2HisWild	3	$1X10^6$ rec2, PO	$1X10^4$ rec2, IP	$1X10^8$ rec2, PO
4a	Rec3	SC+ pNSTrcLux	3	$1X10^6$ rec3, PO	$1X10^6$ rec3, PO	$1X10^8$ rec3, PO
4b	Rec3	SC+ pNSTrcLux	3	$1X10^6$ rec3, PO	$1X10^4$ rec3, IP	$1X10^8$ rec3, PO
5a	Rec4	SC+ pNSTrcDGFP	3	$1X10^6$ rec4, PO	$1X10^6$ rec4, PO	$1X10^8$ rec4, PO
5b	Rec4	SC+ pNSTrcDGFP	3	$1X10^6$ rec4, PO	$1X10^4$ rec4, IP	$1X10^8$ rec4, PO
6a	Neg	SC	3	$1X10^6$ SC, PO	$1X10^6$ SC, PO	$1X10^8$ SC, PO
6b	Neg	SC	3	$1X10^6$ SC, PO	$1X10^4$ SC, IP	$1X10^6$ SC, IP
7a	Pos	Semi-purif. PRRSV	3	40 µg, IM	40 µg, IM	40 µg, IM
7b	Pos	Semi-purif. PRRSV	3	40 µg, IM	40 µg, IM	40 µg, IM

PO: per os; IP: intraperitoneum; IM: intramuscular

Chapter 7

General Conclusions

Porcine reproductive and respiratory syndrome (PRRS) has devastated the swine industry causing approximately half a billion dollars in annual losses in the U.S. (3) due to respiratory and reproductive disease in swine herds. Porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped, single positive-stranded RNA virus, is the causative agent of PRRS. Unknown details of PRRSV replication and pathogenesis as well as problems with the current vaccines, including vaccine failure and reversion to a pathogenic phenotype, demonstrate that there is still a lot of ground to cover when it comes to understanding PRRSV replication and pathogenesis. In this dissertation, we investigated some of these issues.

In the mid 1990s, more virulent forms of PRRSV were identified in herd outbreaks with more severe clinical signs than those observed in a typical PRRSV infection (1, 2). This syndrome was referred to as acute PRRS. To explore the possible mechanism of the emergence of acute PRRS, the open reading frame 5 (ORF5) gene encoding the major envelope protein (GP5) of eight acute PRRSV isolates was characterized. Most of the amino acid substitutions that were identified both amongst the isolates and with other North American isolates were located in the putative signal sequence and two short hypervariable regions at the amino terminus. The ORF5 gene sequence of the acute PRRSV isolate 98-37120-2 from a non-vaccinated swine herd in Iowa was very closely related to that of the RespPRRS MLV and also clustered with the RespPRRS MLV and several Danish isolates that were confirmed to be derived from the

RespPRRS MLV on phylogenetic analyses. Our results showed that the acute PRRSV isolates analyzed in this study differed from each other in ORF5 genes, although they all clustered within the North American genotype. We speculate that the acute isolate 98-37120-2 may be a vaccine-related strain although there is no evidence that the other acute isolates are a reversion of the MLV to a pathogenic phenotype.

To simplify the diagnosis of field isolates in PRRS outbreaks, we developed a heteroduplex mobility assay (HMA) for quickly identifying PRRSV isolates with significant nucleotide sequence identities (≥98%) with the modified live-attenuated vaccines. The major envelope gene (ORF5) of 51 PRRSV field isolates recovered before and after the introduction of the vaccines was amplified, denatured, and reannealed with the HMA reference vaccine strains Ingelvac PRRS MLV and Ingelvac PRRS ATP, respectively. We found that isolates that were identical or nearly identical to the vaccine strain MLV RespPRRS (less than or equal to 2% nucleotide sequence divergence) were all identified by the HMA to form homoduplexes with the reference Ingelvac PRRS MLV. Heteroduplexes were identified with all of the more divergent isolates. The HMA results also correlated well with the results of sequence and phylogenetic analyses. The data indicated that the HMA developed in the study is a rapid and efficient method for large-scale screening of potential vaccine-like PRRSV field isolates for further genetic characterization.

We developed a unique strategy, known as *in vivo* transfection, of infecting pigs with PRRSV by bypassing the traditional *in vitro* cell culture step required for *in vivo* studies. This approach addresses the inherent instability of the PRRSV genome, the requirement of cell culture propagation, and poor virus recovery which have limited the

usefulness of the PRRSV reverse genetics system for *in vivo* studies. We demonstrated that inoculation of RNA transcripts of a PRRSV infectious cDNA clone directly into the lymph nodes and tonsils of pigs produces active PRRSV infection. Using this technology, we more accurately evaluated the quasispecies populations of PRRSV. Earlier studies of PRRSV quasispecies have often begun with a virus stock that had previously been passaged in cell culture, thus increasing the likelihood of unfactored *in vitro* mutations. The *in vivo* transfection technique allowed us to bypass the cell culture passaging step to more accurately characterize the development of quasispecies in pigs. Serum samples collected from pigs that had been inoculated by *in vivo* transfection using the PRRSV infectious cDNA clone pFL12 (4) revealed quasispecies as early as 7 dpi, however, no specific "hot-spots," or mutation-rich regions, were observed at these early time points. We concluded that *in vivo* transfection of PRRSV is a useful method for accurately examining quasispecies populations *in vivo* which has implications for future studies on the mechanism of PRRSV persistence and other aspects of PRRSV replication and pathogenesis.

Evidence of PRRSV vaccine failure and reversion to pathogenic phenotypes clearly demonstrates the need for a newer generation of PRRSV vaccines. We evaluated the ability of *S. choleraesuis* to express the PRRSV major envelope protein, GP5, and tested its immunogenicity in mice. Expression of a 23 kDa protein, which is similar to the predicted size of PRRSV GP5 protein, by *S. choleraesuis* was detected by anti-His serum and a GP5 monoclonal antibody PP5dB4 in western blot analyses although N-terminal sequencing and western blot analyses with other PRRSV antibodies could not definitively confirm the expression. A pilot immunogenicity study with seven groups of

six mice revealed no indication of *Salmonella* replication in the mice or any evidence of antibody production against *S. choleraesuis* or PRRSV GP5. Despite the inability to construct an effective *Salmonella*/PRRSV vaccine, this study has implications for the planning and analysis of future vaccine studies in this area.

In summary, we determined that sequencing and comparison of the ORF5 gene of PRRSV isolates is a useful method for determining the relatedness of PRRSV isolates. A heteroduplex mobility assay, also using the ORF5 gene of PRRSV isolates, is a more rapid and efficient method for differentiating between PRRSV isolates. We also performed *in vivo* transfection using a PRRSV infectious cDNA clone and then used this technology for more accurately evaluating PRRSV quasispecies. Finally, we attempted to develop a Salmonella/PRRSV vaccine using *Salmonella choleraesuis* expressing PRRSV GP5. All of these studies significantly contribute to our knowledge of PRRSV and also demonstrate that a substantial amount of work is still necessary for fully understanding the virus. Future studies investigating the pathogenesis and replication of PRRSV should include manipulation of the infectious clones of PRRSV and the evaluation of its effects *in vivo*. Our *in vivo* transfection technique can be used for such investigations and will greatly facilitate the procedure. Once the specific functions of the genomic regions have been more clearly identified and understood, the mechanistic details of the virus as well as the development of more effective engineered vaccines will certainly be within reach.

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- Opriessnig, T., P.G. Halbur, K.J. Yoon, R.M. Pogranichniy, K.M. Harmon, R. Evans**, K.F. Key**, F.J. Pallares, P. Thomas, and X.-J. Meng. 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. *J Virol*. 76:11837-44.

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Honors and Awards

- 2nd place award (Agricultural and Biological Sciences category) at 17th Annual Graduate Research Symposium of Virginia Tech, March 26, 2001.
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- 1st place award (Basic Science Category) at the 14th Annual VMRCVM Research Symposium, June 6-7 2002.
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- "Genetic characterization and phylogenetic analysis of the ORF5 gene of acute PRRSV isolates." 81st Annual Conference of Research Workers in Animal Diseases, November 2000, Chicago, IL.
- "Genetic basis for the outbreaks of acute porcine reproductive and respiratory syndrome (PRRS)." Virginia Tech Graduate Student Poster session. March 2001.
- "Genetic characterization of the major envelope gene of acute porcine reproductive and respiratory syndrome virus isolates." 2001 VMRCVM Annual Research Symposium, June 2001.
- "Differentiation of vaccine or vaccine-like isolates from field strains of porcine reproductive and respiratory syndrome virus using a heteroduplex mobility assay." 2002 VMRCVM Annual Research Symposium, June 2002.
- "Identification and differentiation of vaccine-like isolates of porcine reproductive and respiratory syndrome virus from virulent field isolates using a heteroduplex mobility assay." 83rd Annual Conference of Research Workers in Animal Diseases, November 2002. St. Louis, MO.
- "Differentiation of vaccine or vaccine-like isolates from field strains of porcine reproductive and respiratory syndrome virus using a heteroduplex mobility assay." Virginia Tech Graduate Student poster session. March 2003.
- "*In vivo* transfection of pigs with RNA transcripts of infectious cDNA clones of PRRS virus: a novel strategy to study *in vivo* PRRSV replication and pathogenesis without having to propagate the virus in cell cultures." 87th Annual Conference of Research Workers in Animal Diseases, December 2006, Chicago, IL.