Micro-epidemiology of PRRS virus infection in vaccinated endemic farms

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Micro-epidemiology of PRRS virus infection in vaccinated endemic farms

Tesis doctoral presentada per **Hepzibar Clilverd Maymo** per optar al grau de Doctor en Veterinària dins el **Programa de Doctorat de Medicina i Sanitat Animals** en el Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del **Dr. Enric M. Mateu de Antonio** i el **Dr. Martí Cortey Marquès**.

FACULTAT DE VETERINÀRIA

UAB, Cerdanyola del Vallès, 2024

El Dr. Enric M. Mateu de Antonio, profesor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de veterinària, i el Dr. Martí Cortey Marquès, profesor agregat del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària,

Declaren,

Que la memòria titulada, "Micro-epidemiology of PRRS virus infection in vaccinated endemic farms" presentada per Hepzibar Clilverd Maymo per a l'obtenció del grau de Doctor en Veterinària, s'ha realitzat sota la seva direcció dins els programa de doctorat de Medicina i Sanitat Animals, i n'autoritzen la seva presentació per tal de ser avaluada per la comissió corresponent.

I, per tal que consti als efectes oportuns, signen el present certificat a Cerdanyola del Vallès.

Dr. Enric Mateu Dr. Martí Cortey Hepzibar Clilverd Maymo

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Abbreviations

ADE: antibody-dependent enhancement

AP: activator protein

APC: antigen-presenting cells

ASVV: American Association of Swine Veterinarians

CMI: cell-mediated immunity

C_t: cycle threshold

CTD: C-terminal domain

CTE: C-terminal extension

CTL: cytotoxic T lymphocyte

DC: dendritic cell(s)

DMV: double-layered membrane vesicle(s)

dsRNA or DNA: double-stranded RNA or DNA

DUB: deubiquitinase

EAV: equine arteritis virus

ELISA: enzyme-linked immunosorbent assay

ELISPOT: enzyme-linked immunosorbent spot

FMIA: fluorescent microsphere immunoassay

GAS: gamma interferon activated site

HIV: human immunodeficiency virus

hpi: hours post-infection

HP-PRRSV: highly pathogenic PRRSV

HS: heparan sulphate

ICTV: International Committee of Taxonomy of Viruses

ID_{50}: infective dose 50

IFA: immunofluorescence assay

IFN: interferon

IFNAR: interferon-alpha/beta receptor

Ig: immunoglobulin

IHC: immunohistochemistry

IκB: inhibitor of nuclear factor kappa beta

IL: interleukin

IPC: internal positive control

IPMA: immunoperoxidase monolayer assay

IRAK: interleukin-1 receptors associated kinase

IRF: interferon regulatory factor

ISG: interferon-stimulated gene

ISGF: interferon-stimulated gene factor

ISH: in-situ hybridization

IV: inactivated vaccine

JAK: Janus kinase

kb: kilobase

LAMP: loop-mediated isothermal amplification

LDV: lactate dehydrogenase-elevating virus

LKD: linker domain

LPS: lipopolysaccharide

LV: Lelystad-virus

mAb: monoclonal antibody

MCMC: Markov chain Monte Carlo

MCREBEL: Management Changes to Reduce Exposure to Bacteria and Eliminates Losses

MDA: maternally-derived antibody(ies)

MERS: Middle East respiratory syndrome coronavirus

MHC: major histocompatibility complex

MID: minimum infectious dose

MLV: modified-live vaccine

miRNA: micro-RNA

MOI: multiplicity of infection

mRNA: messenger RNA

M protein: matrix protein

MSD: Mistery Swine Disease

MyD: Myeloid differentiation primary response.

N protein: nucleocapsid protein

NA: not applicable

NAb: neutralizing antibody(ies)

NendoU: nidovirus-specific uridylate-specific endonuclease.

NF-κB: nuclear factor-kappa beta

NGS: next-generation sequencing

NiRAN: nidovirus RdRp-associated nucleotidyltransferase

NK: natural killer

ns: not significant

nsp: non-structural protein

NTD: N-terminal domain

OD: optical density

ORF: open reading frame

OTU: ovarian tumour domain

OTULIN: ovarian tumour domain deubiquitinase with linear linkage specificity

PAM: porcine alveolar macrophage(s)

PAMP: pathogen-associated molecular pattern

PCP or PLP: papain-like cysteine protease

PCR: polymerase chain reaction

PCR+ve: PCR positive

PCR-ve: PCR negative

PEARS: Porcine Epidemic Abortion and Respiratory Syndrome

PIM: pulmonary intravascular macrophages

Poly I:C: Polyinosinic:polycytidylic acid

pp: polyprotein

PRF: programmed ribosomal frameshifting

PRRS: Porcine reproductive and respiratory syndrome

PRRSV: Porcine reproductive and respiratory syndrome virus

PRRSV-1: *Betaarterivirus suid 1*

PRRSV-2: *Betaarterivirus suid 2*

RdRp: RNA-dependent RNA polymerase

RIG-I: retinoic acid inducible gene-I

RLR: RIG-I-like receptors

RNA: ribonucleic acid

RTC: replication and transcription complex

RT-PCR: reverse transcription polymerase chain reaction.

RT-qPCR: quantitative real-time reverse transcription polymerase chain reaction.

R0: reproduction ratio

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

sgmRNA: subgenomic mRNA

SHVF: simian haemorrhagic fever virus

SIRS: swine infertility and respiratory syndrome

SLA-I: swine leukocyte antigen class I

SLA-II: swine leukocyte antigen class II

S/P ratio: sample to positive control ratio

SP: serine protease

ssRNA or DNA: single-stranded RNA or DNA

STAT: signal transducer and activator of transcription

$TCID_{50}$: 50% tissue culture infectious dose

TLR: toll-like receptor

TM: transmembrane region

TNF-α: tumour necrosis factor alpha

Treg: regulatory T cells

TRIF: toll/Interleukin-1domain receptor-containing adapter protein

TRS: transcription regulatory sequence

TYK: tyrosine kinase

UC: umbilical cord(s)

UTR: untranslated region

VNT: viral neutralisation test

VTE: vertical transmission event(s)

WGS: whole-genome sequencing

woa: weeks of age

ZBD: zinc binding domain

ZF: zinc finger motif

3CLSP: 3C-like serine protease

Summary

The present Ph.D. dissertation aims to delve into understanding the evolutionary dynamics of Porcine reproductive and respiratory syndrome virus (PRRSV), within the framework of farms implementing vaccination. Comprising three studies, it elucidates the virus's evolution in diverse scenarios associated with the infection. The **first study** focuses on the immediate impact shortly after an outbreak of PRRSV in a farrow-to-fattening farm applying a vaccination program. Over the course of a year, piglets of three consecutive batches were monitored from birth to nine weeks of age to assess their PRRSV-status. This involved using RT-qPCR and serological analyses, along with whole genome sequencing (Illumina platform) and Sanger sequencing (nsp2, nsp9, and ORF5), providing an in-depth exploration of the virus's dynamics during the post-outbreak period. Shortly after the outbreak, a notable proportion of sows delivered infected piglets, resulting in a high infection incidence (80%) in nurseries. This initial phase was characterized by a higher genetic diversity, identifying four viral clades, that were attributed to vertical transmission. In the subsequent batch, viral circulation was constrained with 10% cumulative incidence at the end of the nursery period, accompanied by a decline in maternally-derived antibody levels. Remarkably, in the third batch, the infection re-emerged, increasing both vertical transmission events (60%) and cumulative incidence in nurseries (78%); in this case, a single viral variant prevailed. Additionally, the study's findings suggest that non-responder sows and super-spreaders may play a significant role in the transmission of PRRSV. Interestingly, one born-viraemic animal remained viraemic throughout the study but did not contribute to transmission to any other pig. The **second study** covers a two-year observation period of a PRRSV-vaccinated farrow-to-fattening farm that had remained endemic for an extended period. Seven farrowing batches of piglets were followed from birth to nine weeks of age employing RT-qPCR and serological analyses to explore the evolution of the infection. ORF5 and whole-genome sequencing were used to explore the genetic diversity of the virus. These analyses revealed that during the observation period a viral variant emerged and was latter replaced by a new strain introduced laterally. A detailed analysis of the viral variants sought to identify the factors that drive their emergence, encompassing replication kinetics, evasion of neutralization, attachment to macrophages, and inhibition of the interferon response. Notably, the incidence of PRRSV exhibited notable fluctuations in both farrowing units and nurseries over time, correlating with two relevant evolutionary events: the emergence of an escape variant and a lateral

introduction of a new viral strain. The results spotlighted the abrupt replacement of the circulating viral variant by another. The emerging viral variant, with only twenty-five amino acid differences in the whole genome (99.5% nucleotide identity) led to an incidence surge akin to that induced by a new strain, with a 20% nucleotide difference from the previous one. The emerging viral variant harboured most mutations in nsp1α, GP2, GP3, and GP5. Of note, GP5 exhibited an additional glycosylation site in the neutralization epitope and an amino acid deletion. Recombination probably also contributed to the emergence of this viral variant. Moreover, sera from animals exposed to the initially circulating variant had a lower neutralizing capability against the emerging viral variant, suggesting that it was an escape mutant. Besides, the emerging variant showed an increased attachment to macrophages, resulting in an increased yield, but no enhanced ability to inhibit cytokine responses. These findings underscored that the key factors driving the dominance of this variant within the herd were the ability to escape neutralization, coupled with an enhanced capacity to infect macrophages. The **third study** investigated the evolution and persistence dynamics of PRRSV within an endemically infected and vaccinated farm that was approaching stabilization. Over an eight-month period, piglets from three farrowing batches were followed from birth to nine weeks of age, examining the incidence of the disease by RT-qPCR and the serological status of the animals. Sequencing techniques were used to determine the transmission and evolution of the virus. When the farm was close to achieve stabilization, a critical event unfolded: a single failure in a recall vaccination of sows related to high temperatures in summer led to a resurgence of the infection. This vaccination lapse resulted in a surge to almost 100% incidence at six weeks of age, a drop in maternally-derived immunity, and a rise in genetic diversity within the virus. Notably, prior to this vaccination issue, transmission was relatively controlled, and the genetic diversity of the virus was declining. Furthermore, the study reveals that both selection and recombination contributed to the viral diversity observed in this farm. Ultimately, reestablishing the vaccination program led to a PRRSV-stable status, underscoring the importance of strict vaccination adherence to control PRRSV incidence and genetic diversity. Taken together, the findings of this Ph.D. dissertation advance our understanding of the mechanisms underlying PRRSV persistence and its evolution within farms under vaccination pressure. The results also highlight PRRSV's plasticity to adapt and persist in herds, emphasizing the challenges in controlling PRRSV and underscoring the critical need for developing effective vaccines and eradication strategies.

Resumen

La presente tesis doctoral tiene como objetivo profundizar en la comprensión de la dinámica evolutiva del virus del síndrome reproductivo y respiratorio porcino (PRRSV), en el marco de las granjas que implementan vacunación. La tesis consta de tres estudios, destinados a averiguar la evolución del virus en varios escenarios asociados a la infección. El **primer estudio** se centra en el impacto inmediato poco después de un brote de PRRSV en una granja de reproductoras y transición, que aplicaba un programa de vacunación en las cerdas. En el transcurso de un año, se monitorearon lechones de tres lotes consecutivos desde el nacimiento hasta las nueve semanas de edad, para evaluar su estado de infección frente al PRRSV utilizando RT-qPCR y análisis serológicos, junto con la secuenciación del genoma completo (plataforma Illumina) y secuenciación Sanger (nsp2, nsp9 y ORF5). En conjunto, esto permitió un análisis en profundidad de la dinámica del virus durante el período posterior al brote. Poco después del brote, una proporción notable de cerdas parieron lechones virémicos, que resultó en una elevada incidencia de infección (80%) en las transiciones. Esta fase inicial se caracterizó por una mayor diversidad genética, identificando cuatro clados del virus, cuyo origen se atribuyó a la transmisión vertical. En el lote posterior, la circulación vírica disminuyó hasta un 10% de incidencia acumulada al final del período de transición. De forma paralela, se produjo una caída de los niveles de anticuerpos de origen materno presentes en los lechones. En el tercer lote, la infección resurgió, aumentando tanto los eventos de transmisión vertical (60%) como la incidencia acumulada en las transiciones (78%). En ese tercer lote sólo circulaba una única variante vírica. Además, los resultados del estudio sugieren que existían cerdas que no desarrollaron una inmunidad efectiva a pesar de haber sido vacunadas en repetidas ocasiones, transmitiendo verticalmente la infección a su progenie en sucesivas gestaciones. También se observó la existencia de animales superdiseminadores, que pueden tener un papel importante en la transmisión del PRRSV. Curiosamente, un animal que nació virémico y se mantuvo virémico durante todo el estudio, no contribuyó a la transmisión a ningún otro cerdo. El **segundo estudio** cubre un período de observación de dos años de una granja que tenía reproductoras y transiciones y que había permanecido endémica de PRRSV durante un período prolongado, a pesar de implementar vacunación. Con el objetivo de examinar la evolución de la infección, se siguieron siete lotes de lechones desde el nacimiento hasta las nueve semanas de edad mediante RT-qPCR y serología. Se examinó la diversidad genética del virus circulante mediante la secuenciación de ORF5 y del genoma completo. Estos

análisis revelaron que durante el período de observación surgió una variante vírica que fue sustituida por una nueva cepa introducida lateralmente. Se llevó a cabo un análisis pormenorizado de las variantes virales con el propósito de identificar los factores que podrían haber impulsado su aparición, incluyendo aspectos como la cinética de replicación, la evasión de neutralización, la capacidad de adherencia a macrófagos y la inhibición de la respuesta de interferón. La incidencia del PRRSV mostró fluctuaciones notables tanto en las maternidades y transiciones a lo largo del tiempo, correlacionándose con dos acontecimientos evolutivos relevantes: la aparición de una variante de escape y una introducción lateral de una nueva cepa vírica. Los resultados pusieron de manifiesto la sustitución brusca de la variante vírica circulante por otra. Además, la variante vírica emergente, con sólo veinticinco diferencias de aminoácidos en todo el genoma (99,5% de identidad de nucleótidos) provocó un aumento de incidencia similar al inducido por la introducción de una nueva cepa que tenía una similitud nucleotídica de tan solo el 80% respecto de la anterior. En la variante vírica emergente, las mutaciones se concentraban en la nsp1α, GP2, GP3 y GP5. Cabe destacar que la GP5 mostró un sitio de glicosilación adicional en el epítopo de neutralización y una deleción. La recombinación probablemente también contribuyó a la aparición de esa variante vírica. Además, los sueros de animales expuestos a la variante que circulaba inicialmente tenían una menor capacidad neutralizante contra la variante vírica emergente, sugiriendo que se trataba de un mutante de escape. Por otra parte, la variante emergente mostró un aumento de su capacidad de adhesión a los macrófagos, dando lugar a un aumento del título vírico, pero sin mostrar mayor capacidad de inhibición de la respuesta de citocinas. Estos hallazgos señalaban que los factores clave que impulsaron el predominio de esta variante fueron la capacidad de escapar de los anticuerpos neutralizantes, junto con una mayor capacidad de infectar a los macrófagos. El **tercer estudio** investigó la dinámica de evolución y persistencia del PRRSV dentro de una granja vacunada e infectada de forma endémica, que se estaba acercando a la estabilización. A lo largo de un período de ocho meses, se siguieron lechones de tres lotes desde el nacimiento hasta las nueve semanas de edad y se examinó la incidencia de la enfermedad mediante RT-qPCR así como el estado serológico de los animales. El uso de técnicas de secuenciación permitió analizar la transmisión y evolución del virus. Cuando la granja estaba cerca de conseguir la estabilización, se produjo un acontecimiento crítico: un fallo en una vacunación de recuerdo de las cerdas, relacionado con las altas temperaturas del período estival, provocó un resurgimiento de la infección.

Este fallo de vacunación dio lugar a un aumento de casi el 100% de incidencia en las seis semanas de edad, una caída de la inmunidad maternal y un aumento de la diversidad genética del del virus. Destacablemente, antes del problema de vacunación, la transmisión estaba relativamente controlada y la diversidad genética del virus estaba disminuyendo. Además, el estudio revela que tanto la selección como la recombinación contribuyeron a la diversidad genética del virus observada en esa granja. En última instancia, el restablecimiento del programa de vacunación condujo a la estabilización de la infección por PRRSV en la granja, subrayando la importancia de seguir estrictamente los programas vacunales para controlar la incidencia de PRRSV y la diversidad genética. En conjunto, esta tesis doctoral nos permite avanzar en la comprensión de los mecanismos subyacentes a la persistencia del PRRSV y su evolución en granjas bajo presión de vacunación. Asimismo, los resultados destacan la plasticidad del PRRSV para adaptarse y persistir en las granjas, poniendo de manifiesto los retos en el control del PRRSV y subrayando la necesidad de desarrollar vacunas y estrategias de erradicación más efectivas.

Resum

La present tesi doctoral té com a objectiu aprofundir en la comprensió de la dinàmica evolutiva del virus de la síndrome reproductiva i respiratòria porcina (PRRSV), en el marc de les granges que implementen vacunació. La tesi consta de tres estudis, destinats a esbrinar l'evolució del virus en diversos escenaris associats a la infecció. El **primer estudi** es centra en l'impacte immediat poc després d'un brot de PRRSV en una granja de reproductores i transició, que aplicava un programa de vacunació a les truges. Al llarg d'un any, es van seguir garrins de tres lots consecutius des del naixement fins a les nou setmanes d'edat per avaluar el seu estat d'infecció enfront del PRRSV, utilitzant RT-qPCR i anàlisis serològiques, juntament amb la seqüenciació del genoma sencer (plataforma Illumina) i la seqüenciació Sanger (nsp2, nsp9 i ORF5). En conjunt, això va permetre un anàlisi en profunditat de la dinàmica del virus durant el període posterior al brot. Poc després del brot, una proporció notable de truges van parir garrins virèmics, que va conduir a una alta incidència d'infecció (80%) a les transicions. Aquesta fase inicial es va caracteritzar per una major diversitat genètica, identificant-se quatre clades virals, l'origen dels quals es va atribuir a la transmissió vertical. En el lot posterior, la circulació vírica va disminuir al 10% d'incidència acumulada al final del període de transició. De forma paral·lela es va produir una caiguda dels nivells d'anticossos maternals presents en els garrins. En el tercer lot, la infecció va ressorgir, augmentant tant els esdeveniments de transmissió vertical (60%) com la incidència acumulada en les transicions (78%). En aquest tercer lot només circulava una única variant vírica. A més, els resultats de l'estudi suggereixen que hi havia truges que no van desenvolupar una immunitat efectiva malgrat haver estat vacunades en repetides ocasions, transmetent verticalment la infecció en successives gestacions. També s'observa l'existència de super-disseminadors, que poden tenir un paper important en la transmissió del PRRSV. Curiosament, un animal que va néixer virèmic i es va mantenir virèmic durant tot l'estudi, no va contribuir a la transmissió a cap altre porc. El **segon estudi** cobreix un període d'observació de dos anys d'una granja que tenia reproductores i transicions i que s'havia mantingut endèmica de PRRSV durant un període prolongat, malgrat implementar vacunació. Amb l'objectiu d'examinar l'evolució de la infecció, es van seguir set lots de garrins des del naixement fins a les nou setmanes d'edat mitjançant RT-qPCR i serologia. La diversitat genètica del virus es va examinar emprant la seqüenciació d'ORF5 i de genoma sencer. Aquestes anàlisis van revelar que durant el període d'observació va sorgir una variant vírica que va ser

substituïda per una nova soca introduïda lateralment. Es va realitzar una anàlisi detallada de les variants virals amb la finalitat d'identificar els factors que impulsen la seva aparició, incloent aspectes com la cinètica de replicació, l'evasió de neutralització, la capacitat d'adherència a macròfags i la inhibició de la resposta d'interferó. La incidència del PRRSV va mostrar fluctuacions notables tant a les maternitats i transicions al llarg del temps, correlacionant-se amb dos esdeveniments evolutius rellevants: l'aparició d'una variant d'escapament i una introducció lateral d'una nova soca vírica. Els resultats van posar de manifest la substitució brusca de la variant viral circulant per una altra. A més, la variant vírica emergent, amb només vint-i-cinc diferències d'aminoàcids en tot el genoma (99,5% d'identitat de nucleòtids), va provocar un augment d'incidència semblant al produït per la introducció d'una nova soca amb una identitat nucleotídica de només el 80% respecte de la soca anterior. En la variant emergent, les mutacions es concentraven a la nsp1α, GP2, GP3 i GP5. Cal destacar que GP5 contenia un lloc de glicosilació addicional a l'epítop de neutralització i una deleció. La recombinació probablement també va contribuir a l'aparició d'aquesta variant vírica. A més, els sèrums d'animals exposats a la variant que circulava inicialment tenien una menor capacitat neutralitzant contra la variant vírica emergent, suggerint que es tractava d'un mutant d'escapament. Per altra banda, la variant emergent va mostrar un augment de la seva capacitat d'adhesió als macròfags, donant lloc a un augment del títol víric, però sense mostrar major capacitat d'inhibició de la resposta de citocines. Aquestes troballes assenyalaven que els factors clau que van impulsar el predomini d'aquesta variant van ser la capacitat d'escapar dels anticossos neutralitzants, juntament amb una capacitat més gran d'infectar els macròfags. El **tercer estudi** va investigar la dinàmica d'evolució i persistència del PRRSV dins d'una granja vacunada i infectada de manera endèmica, que s'estava apropant a l'estabilització. Durant un període de vuit mesos, es van seguir garrins de tres lots de parts des del naixement fins a les nou setmanes d'edat i es va examinar la incidència de la malaltia mitjançant RT-qPCR així com l'estat serològic dels animals. L'ús de tècniques de seqüenciació va permetre determinar la transmissió i l'evolució del virus. Quan la granja estava a prop d'assolir l'estabilització, es va produir un esdeveniment crític: una fallida en una vacunació de record de les truges, relacionada amb les altes temperatures del període d'estiu, va provocar un ressorgiment de la infecció. Aquest lapse de vacunació va provocar un augment de gairebé el 100% d'incidència a les sis setmanes d'edat, una caiguda de la immunitat maternal i un augment de la diversitat genètica dins del virus. Remarcablement, abans d'aquest problema de vacunació, la

transmissió estava relativament controlada i la diversitat genètica del virus tenia una tendència decreixent. A més, l'estudi revela que tant la selecció com la recombinació van contribuir a la diversitat genètica del virus observada en aquesta granja. En última instància, el restabliment del programa de vacunació va conduir a l'estabilització de la infecció per PRRSV de la granja, subratllant la importància de l'adhesió estricta als programes vacunals per controlar la incidència de PRRSV i la diversitat genètica. En conjunt, aquesta tesi ens permet avançar en la comprensió dels mecanismes subjacents a la persistència del PRRSV i la seva evolució a les granges sota pressió de vacunació. Tanmateix, els resultats posen de manifest la plasticitat del PRRSV per adaptar-se i persistir en les granges, emfatitzant els reptes en el control del PRRSV i subratllant la necessitat de desenvolupar vacunes i estratègies d'erradicació més efectives.

PART I:

Thesis background

Chapter 1.

Introduction

1.1. INTRODUCTION AND JUSTIFICATION

Porcine reproductive and respiratory syndrome (PRRS) is a disease that holds great economic importance in the global swine industry. Its impact is felt in the realms of animal health, welfare, and productivity, and has become one of the costliest diseases in pig production. PRRS virus (PRRSV) targets macrophages, and despite causing systemic infection, the clinical manifestations target primarily the reproductive functionality and the respiratory system, resulting in reproductive failure and pneumonia. Given the deep interaction of the virus with the immune system, secondary infections frequently arise in young pigs.

The genetic diversity of PRRSV is a key challenge in the control and prevention of its infection. Like other positive-sense single-stranded RNA viruses, PRRSV exhibits a high mutation rate, and recombination between isolates is a common occurrence, continually giving rise to novel variants. Genetic differences among PRRSV isolates are linked to differences in virulence, pathogenesis, and induced immune response, thereby complicating the diagnosis and control of the disease. Genetic diversity is suggested to also contribute to PRRSV persistence within herds. Unfortunately, a precise map of the genetic determinants is not available yet. Therefore, understanding the genetic differences, molecular epidemiology, and evolution of PRRSV is imperative for understanding the epidemiology of the infection and developing targeted and effective control measures, including the development of vaccines.

Moreover, PRRSV can serve as an advantageous model for investigating the molecular mechanisms that underlie RNA virus evolution and adaptation. Given its small genome, high mutation rate, and intricate replication cycle, PRRSV is an ideal system for such studies.

In summary, examining PRRSV evolution and adaptation can provide insights into the epidemiology and molecular mechanisms governing viral evolution and transmission, which could have implications for the control and prevention of other RNA virus infections.

1.2. BRIEF HISTORY OF PRRSV

Since the mid-1980s, severe outbreaks of a previously unseen disease that were marked by significant reproductive failure in sows and pneumonia, reduced growth rate, and heightened mortality in piglets were documented in the United States[1–3]. The disease was initially referred to as “Mistery Swine Disease” (MSD) or “Swine Infertility and Respiratory Syndrome” (SIRS) due to its unknown nature [4].

Similar outbreaks were likewise documented in Germany in 1990[5,6]. It would later be discovered that two closely related species of the same genus of viruses had emerged simultaneously on two continents causing the same disease. The disease spread rapidly across Europe and received various names, such as "abortus blauw” (blue abortion) in Netherlands, "blue-eared pig disease" in the United Kingdom, and “`Seuchenhafter Spatabort der Schweine” (Porcine Epidemic Abortion and Respiratory Syndrome (PEARS)) in Germany[6–11].

PRRS could not be contained neither in North America nor in Europe and spread to most pig producing countries. In Asia, an initial wave of PRRSV infection arrived very early because of trading with the United States, with documented outbreaks dating back to 1987 in Japan[12–14] and to 1992 in Taiwan[15]. At present PRRSV is considered endemic in most pork-producing countries worldwide and, once endemically established, only two countries have been able to eradicate the infection at some point: Chile and Hungary [16,17].

The etiological agent of PRRS was first isolated in 1991 in the Central Veterinary Institute in Lelystad, the Netherlands[18] and in 1992 by Collins et al.[19] in the United States. Both isolates became the reference strains for the European and North American viruses, named Lelystad-virus (LV) and VR-2332 strains, respectively[18,19]. PRRSV was determined to be a new RNA virus classified in to the genus *Arterivirus*[20]. In 1992, the current name of the disease and of the etiological agent, Porcine Reproductive and Respiratory Syndrome (PRRS) and PRRS virus, respectively, was established in the first International Symposium on SIRS held in Saint Paul (Minnesota, United States).

Although closely related, both European and American viruses were different genetically and antigenically, being initially classified as two genotypes within a single *Porcine reproductive and respiratory syndrome* species: the European or type 1 genotype,

and the North American or type 2 genotype[21–24]. The nucleotide sequence similarity between both when whole genome sequences are compared is around 55%-60%[24–27].

The origin of PRRSV remains unknown. The virus had already been circulating within the porcine population before the disease was detected. According to retrospective serological tests, the first pigs seropositive for PRRSV found in North America are dated back to 1979 and to 1987 in Eastern Germany[28,29]. It has been postulated that both genotypes derive from a common ancestor that existed from a decade to over a century before the occurrence of the initial clinical instances, since when they evolved independently, until emerging almost simultaneously in the two continents, an exceptional occurrence[27,30–33]. Stadejek et al.[34,35] suggested that the genetic diversity and distribution observed in PRRSV-1 in Europe and Russia could be indicative of an Eastern European origin, and was later introduced into Western Europe, subsequently spreading through livestock trade. Additionally, it has been suggested that this common ancestor could come from another host species[32,36]. Plagemann[36] proposed that PRRSV may have arisen from an ancestor similar to the lactate dehydrogenase-elevating virus (LDV) due to a species jump from rodents to wild boars from Eastern Europe. The importation of infected European wild boars into the United States in 1912, coupled with the parallel independent evolution of the viruses on both continents, could potentially account for the divergence between North American and European PRRS viruses. However, this cross-species transmission theory is feeble owing to the lack of evidence and the absence of intermediary viruses identified in mice or wild boars[37–39]. There is no fully accepted hypothesis by now.

1.3. TAXONOMY AND CLASSIFICATION

Nowadays, PRRSV is classified within the genus *Betaarterivirus* in the *Arteriviridae* family within the order *Nidovirales* that includes other three families: *Coronaviridae*, *Mesoniviridae*, and *Roniviridae*[40,41]. *Nidovirales* are enveloped positive-strand RNA viruses that share a common genome organization and proteins implicated in RNA replication and transcription. The term "Nidovirales" derives from the word "nidus" in Latin, which denotes the "nested" set of 3' co-terminal subgenomic mRNAs generated during gene expression[40,42].

The *Arteriviridae* family includes also equine arteritis virus (EAV), which gave name to this family, lactate dehydrogenase-elevating virus (LDV), and simian haemorrhagic fever virus (SHFV), among others[40–42]. Arteriviruses are characterized by their small genome size which ranges from 13-16 kilobases (kb), a virion diameter of 40-60 nm, and a particular narrow host range and cell-tropism, primarily targeting cells of the monocyte/macrophage lineage and dendritic cells, but also endothelial cells[40,42,43].

According to the classification approved by the International Committee on Taxonomy of Viruses (ICTV), the previously PRRSV genotypes are currently classified into two species: *Betaarterivirus suid 1* or PRRSV-1 (formerly the European genotype), and *Betaarterivirus suid 2* or PRRSV-2 (formerly the North American genotype), and categorized into two different subgenuses, *Eurpobartevirus* and *Ampobartevirus*, respectively[41,44] (Figure 1).

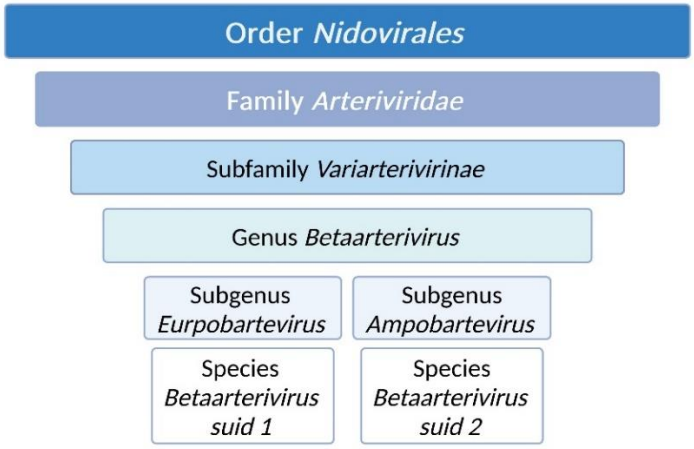

Figure 1. Taxonomy of PRRSV according to ICTV. Created with BioRender.com.

Based on the sequencing and phylogenetical analyses of open reading frame (ORF) 5 and ORF7, PRRSV-1 and PRRSV-2 are further differentiated into distinct subtypes or lineages. Concerning PRRSV-1, four subtypes were originally identified. Subtype 1, also called pan-European subtype, is predominantly found in Central and Western European countries, although it has been reported in Asia and North America[45–51]. Subtypes 2 and 3 have only been isolated in Eastern European countries and encompass strains of diverse virulence, including highly virulent strains like Lena or SU1-bel. Subtype 4 comprises strains originated from Latvia and Belarus[34,35,52]. Shi et al.[38] further divided PRRSV-1 subtype 1 strains into 12 sub-clades established on inter-clade genetic distances. More

recently, Balka et al.[53] proposed three lineages within subtype 1 and lineages 1 and 3 subdivided into clades 1A-1G and 3A-3G, respectively. None of the classifications below the level of subtype have reached a consensus. On the other hand, nine lineages have been defined for PRRSV-2, of which the four larger lineages (1, 5, 8, and 9) are further divided into sub-lineages[54]. Recently, Yim-im et al.[55] updated PRRSV-2 classification into eleven lineages and 21 sub-lineages.

In recent years, the emergence of PRRSV virulent strains has become a significant concern in the global swine industry. The first highly virulent strains were documented for PRRSV-2 in the United States in 1995 (strain VR-2385)[56,57] and 2001 (strain MN184)[26]. Those highly virulent strains were reported to cause more severe outbreaks, including mortality in sows[58]. In 2006, a new PRRSV-2 of unparalleled virulence was described in China[59]. That strain resulted in a fatality rate that could easily reach 25%, and produced clinical signs and lesions rarely seen with other PRRSV strains, such as haemorrhagic pneumonia, petechiae in the kidneys, haemorrhagic lymph nodes, gall bladder oedema, etc. Since then, several virulent strains belonging to PRRSV-2 have been identified in both regions, such as SDSU 73, JA 142, MN184, NADC30, NADC34, ISU-1, ISU-5, and 1-4-4c in United States, and NADC30-like, 14LY01-FJ, 14LY02-FJ 15LY01-FJ, and 15LY02-FJ in Asia[60–64].

Nevertheless, strains of PRRSV-1 with high virulence have also been reported, such as PRRSV-1.3. Lena[65] and SU1-bel[66], and PRRSV-1.2. BOR59 strains[67] in eastern Europe, as well as subtype 1.1 strains like PR40 in Italy[68] and AUT15-33 in Austria[69]. In 2020, a new highly virulent PRRSV-1 strain emerged in Spain causing severe outbreaks marked by elevated abortion rates and mortality in sows, along with high mortality rates in weaners and growers. This strain originated from the recombination between a PR40-like strain with other PRRSV-1 local Spanish isolates[70,71].

Although there is no agreed-upon nomenclature to refer to these strains (atypical, with atypical virulence, virulent, highly virulent, pathogenic, highly pathogenic, etc.), the term "virulent" has been proposed to generally refer to them[72].

1.4. PRRSV, THE VIRUS

1.4.1. PRRSV virion.

PRRSV virions are pleomorphic, varying from round to oval, with a diameter between 50-65 nm[73]. The viral particles possess an envelope with a very smooth surface, formed by an external lipid bilayer and the short ectodomains of the abundant GP5/M heterodimer complexes, with a few protrusions that correspond to the ectodomains of the GP2/3/4 heterotrimer complexes. Enclosed within the virions is a double-layered core with an average diameter of 39 nm, which is comprised of two layers of N proteins that interact with the genomic RNA and are tightly packed forming a hollow ball[73,74]. Figure 2 illustrates PRRSV viral particle.

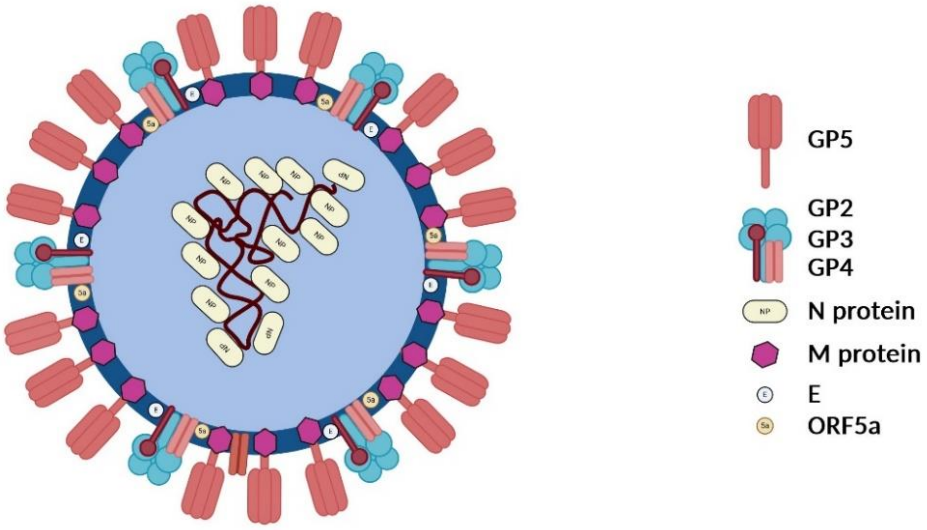

Figure 2. PRRS viral particle. GP: glycoprotein; M protein: matrix protein; N protein: nucleocapsid protein; ORF: open reading frame. Created with BioRender.com.

1.4.2. Physic-chemical properties of PRRSV.

Similar to other enveloped RNA viruses, PRRSV is susceptible to inactivation by organic solvents, desiccation, and extreme pH values, either too acidic or too alkaline. Benfield et al.[75] showed that the virus loses infectivity rapidly at pH levels below 4 or above 7. Additionally, viral viability diminishes rapidly with increasing temperatures. While it can remain viable for months or years at -70°C, inactivation occurs in 6 min at

56°C. Stability at 4°C lasts for 140 h[76]. In tissues of infected animals, the virus also undergoes rapid degradation and, 72 h after death, most samples turn negative[77]. Common disinfectants, such as iodine, chlorine, or quaternary ammonium, are very effective[78].

1.4.3. Genomic organization of PRRSV.

PRRSV genome spans approximately 15 kb in size. It contains a 5' cap structure and a 3' polyadenylated end, and two untranslated regions (UTR) that flank the genome at its both termini. PRRSV genome encodes at least 10 ORF that give rise to 8 structural and at least 16 non-structural proteins (further reviewed [27,43,74,79]).

After the 5'-UTR, ORF1a and ORF1b can be found, constituting 75% of the entire genome. These ORFs encode two large polyproteins, namely pp1a and pp1ab. Both polyproteins undergo viral protease-mediated cleavage into 16 non-structural proteins, specifically nsp1 to nsp12, with both nsp1 and nsp7 further cleaved to nsp1α/β and nsp7α/β, plus two proteins encoded after a frameshift, nsp2TF and nsp2N[27,43,80–82].

Further along in the translation direction are the remaining ORFs from 2 to 7, which encode for the major and minor structural proteins. The major structural proteins comprise the glycoprotein 5 (GP5), the matrix protein (M), and the nucleocapsid protein (N). The minor structural proteins of PRRSV are present in smaller amounts and include three N-glycosylated proteins, GP2, GP3, and GP4, which form a trimer, and two non-glycosylated proteins, E or 2b and ORF5a[43]. Figure 3 depicts de genome organization of PRRSV.

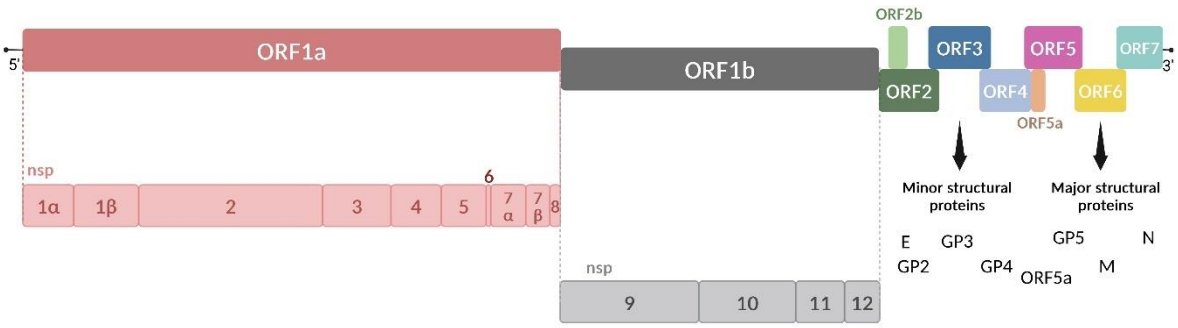

Figure 3. Genome organization of PRRSV. GP: glycoprotein; nsp: non-structural protein; ORF: open reading frame. Created with BioRender.com.

1.4.4. PRRSV replication.

1.4.4.1. Cell tropism, receptors, and entry mediators.

PRRSV has a restrictive cellular tropism for $CD163^+$ cells[83]. CD163 is expressed in macrophages, particularly porcine alveolar macrophages (PAM), but also in other macrophages in the lungs, lymphoid tissues, and placenta, and in dendritic cells (bone marrow-derived and monocyte-derived dendritic cells (DC))[84–86]. Furthermore, PRRSV also replicates *in vitro* in non-porcine cell lines, namely the African green monkey kidney cell line MA-104 and its derived MARC-145 and CL2621 cells[19,87]. It can also replicate in originally non-permissive cell lines transfected to express PRRSV receptors[88,89]. Some permissive macrophage cell lines have been produced as well [90,91].

The only essential cellular receptor identified up to now is the CD163[83,92–95]. Genetic edition of CD163 by modifying or deleting the domain 5 of the protein has been shown to abolish susceptibility to PRRSV[96–99]. Besides CD163, other co-receptors and entry factors have been identified (Table 1), and several reviews have dealt with these and their specific roles[100–102]. Recently, it has been proposed that macropinocytosis can have an important role in the infection of macrophages[102–104].

Table 1. Other co-receptors and entry factors for PRRSV.

Molecule	Role	References
Heparan sulphate (HS)	Initial virion attachment and accumulation on cell surface through interaction with M or M/GP5.	105,106
Porcine sialoadhesin, CD169, SIGLEC-1	Virion attachment and endocytosis through interaction with GP5/M.	92,106–108
SIGLEC-10	Complementary sialoadhesin involved in PRRSV entry into PAM.	109,110
Vimentin	Main PRRSV receptor in cell lines of fibroblastic origin. It interacts with N protein.	111,112
Aspartic protease cathepsin E	Cleavage of GP5 during fusion of the viral envelope with the cell membrane.	113,114
Non-muscle myosin heavy chain 9 (MYH9)	Indispensable co-factor to facilitate virion endocytosis.	115–117
CD151	Interaction with RNA 3'-UTR during virus uncoating.	118–120
DC-SIGN (CD209)	Unclear.	121,122

1.4.4.2. Virus attachment, internalization, and disassembly.

PRRSV infection is initiated through a receptor-mediated endocytosis. The virus first establishes contact with the host cell by means of the interaction between GP5/M heterodimer and the heparan sulphate (HS) of the cell (Figure 4). The binding of HS to PRRSV M and to GP5/M complex is not specific, nor strictly required for infection, but it allows for the accumulation of viral particles on the cell surface. In turn, this facilitates and stabilizes the interaction between the virus and the higher affinity receptor CD169 (porcine sialoadhesin), hence enhancing the attachment and internalisation of viral particles into the host cell[86,105,106]. The following step is the interaction between GP5/M and the porcine sialoadhesin on the cell surface, in which the N-terminal structural domains of the sialoadhesin binds specifically to sialic acids in GP5[106–108,123,124]. This triggers viral internalization via clathrin-mediated endocytosis[106,107,124]. Nevertheless, the binding of sialoadhesin allows internalization but it is not sufficient for productive infection[106,107].

Once PRRSV binds to sialoadhesin, the virus undergoes internalization through the formation of a clathrin-coated endocytic vesicle. Within the early endosomes, the GP2/GP3/GP4 heterodimer, particularly GP2 and GP4, interacts with CD163[93,94]. The interaction with CD163, together with a pH drop, mediates the virion uncoating. Following endosome acidification and membrane fusion with the endosome envelope, the virion undergoes uncoating, allowing for the subsequent release of the viral genome into the cytoplasm and the completion of the viral internalisation[92,94,95,125]. While HS and porcine sialoadhesin are dispensable for PRRSV infection, CD163 is the essential receptor[83,107,126,127]. Additionally, the E protein acts as an ion channel and seems to participate in the uncoating and release of viral genome into the cell's cytoplasm. The E protein undergoes conformational changes in the acidic conditions within the endosome, and this, in turn, allows ions to enter the virion and facilitate the nucleocapsid breakdown[128].

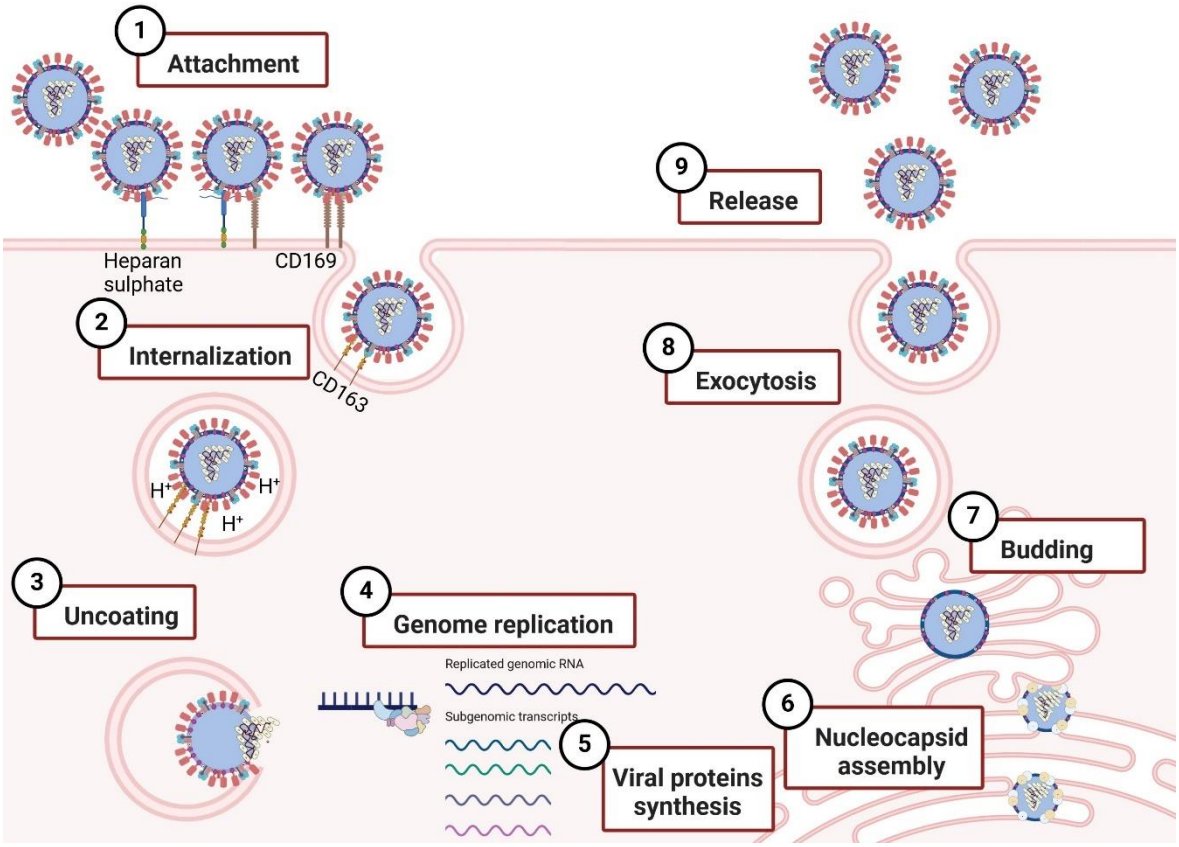

Figure 4. Overview of PRRSV infection cycle. After initial attachment, the PRRSV virion undergoes internalization through clathrin-mediated endocytosis. Within the endosome, interaction with CD163, along with acidification, leads to uncoating and the release of the viral genome into the cytoplasm, initiating then viral genome replication. The newly generated viral genomes and N proteins assemble into the nucleocapsid, which is enveloped in the smooth endoplasmic reticulum or Golgi complex. Ultimately, the new virions are released via exocytosis. Adapted from Lunney et al. [129]. Created with BioRender.com.

1.4.4.3. Genome replication, assembly, and budding.

Replication starts when the viral genome is delivered into the cytoplasm after uncoating and decapsidation. Given the positive sense genome of the virus, ORF1a and ORF1b undergo immediate translation resulting in the formation of the polyproteins pp1a and, through a -1 ribosomal frameshift before the end of ORF1a, pp1b[130,131]. These polyproteins are subsequently processed by internal proteases into various nsps, including the RNA-dependent RNA polymerase (RdRp), the RNA helicase, and the endoribonuclease, among others, that come together to form the replication and transcription complex (RTC)[43,80,130,131].

In the perinuclear region, nsps from the pp1a modify the endoplasmic reticulum resulting in the formation of double-layered membrane vesicles (DMV). It is in those DMV where RTC assembles, is anchored and, thereby, accommodates viral RNA synthesis[130–134]. This RTC kickstarts the synthesis of full-length and sub-genome-length complementary minus strands RNA[27,81]. Following this, the full-length strand synthesis acts as a template for genomic RNA, while the discontinuous strand synthesis serves as a template to produce a nested set of subgenomic mRNA (sgmRNA). These sgmRNA are responsible for translating the structural proteins of the virion[27,42,81]. The new genomes are then packed within the nucleocapsid formed by phosphorylated N protein, which results in the formation of new virion cores[135,136]. Refer to Figure 5 for an overview of the PRRSV replication cycle.

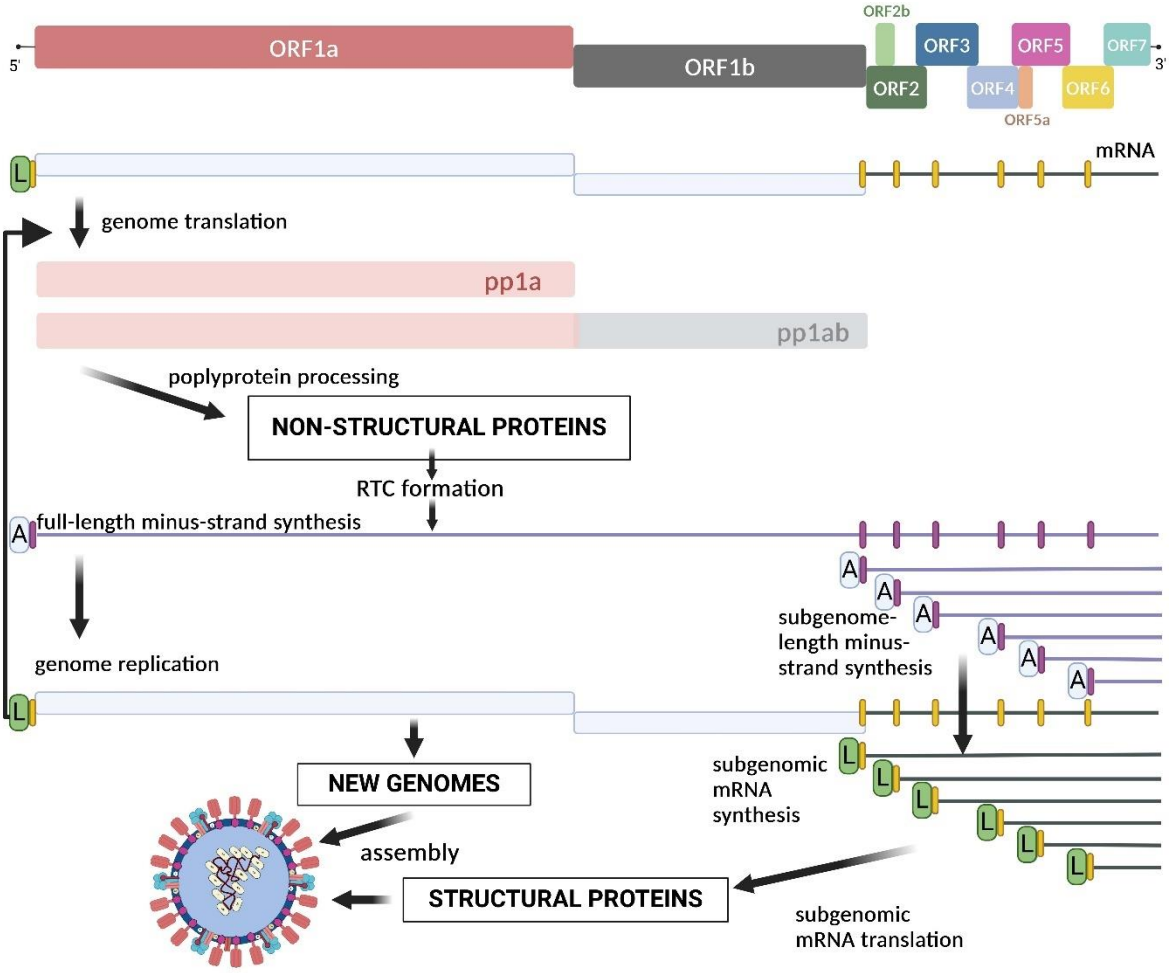

Figure 5. Replication cycle of PRRSV. Once the PRRSV positive-sense genome is released into the cytoplasm, direct translation by the ribosome results in polyproteins pp1a and pp1ab, which are cleaved into the fourteen non-structural proteins. These assemble into the replication and transcription complex (RTC), which starts the synthesis of the full-length and discontinuous complementary minus strands RNA from the 3' end. The full-length minus-strand serves as

a template for genomic RNA. The discontinuous strand synthesis generates subgenomic-length minus strands that serve as templates for subgenomic mRNA synthesis, which then are translated into the structural proteins of the virus. The new genomes and structural proteins are assembled into new virions. Transcription regulatory sequences (TRS) are coloured in yellow. A: 3' anti-leader; L: 5' leader; RNA: ORF: open reading frame; pp: polyprotein; RTC: replication and transcription complex; Adapted from Fang and Schnider[81]. Created with BioRender.com

The forming virion buds into the lumen of the smooth endoplasmic reticulum or Golgi complex and is then enveloped by membranes derived from these structures[40]. The viral envelope proteins are retained in the envelope. GP5 and M form heterodimers that are essential for the viral assembly and structure[137,138]. The minor structural proteins, which are not required for assembly, interact with each other along with the E protein[138]. While the virion proceeds through the Golgi apparatus, the viral glycoproteins undergo glycosylation[139]. Finally, the newly formed virions are released from the cell via the exocytic pathway[43].

1.4.5. Functions of the non-structural proteins.

The function of the non-structural proteins in PRRSV is not fully known. Some of the nsps have been recognized as essential components of viral replication and transcription machinery, while some have been shown to be involved in the modulation of host immune response and immune evasion (further reviewed[140,141]). The subsequent section provides an overview of the distinct roles of the nsps, with their primary functions summarized in Table 2.

Table 2. Main functions of PRRSV non-structural proteins (nsp).

Gene	Protein	Main functions and characteristics
ORF1a	nsp1α	Processing of the polyprotein pp1a. Regulator of sgmRNA synthesis. Inhibition of type I IFN production. Downregulation of TNF-α. Induction of non-neutralizing antibody response.
	nsp1β	Processing of the polyprotein pp1a. Induction of nsp2TF and nsp2N production. Inhibition of type I IFN production and signalling. Downregulation of TNF-α. Induction of non-neutralizing antibody response.

	nsp2	Highly variable protein. Processing of the polyprotein pp1a and co-factor of nsp4. DMV formation and RTC assembly. Inhibition of type I IFN production. Downregulation of TNF-α. Induction of non-neutralizing antibody response.
	nsp2TF	Processing of the polyprotein pp1a and suppression of innate immune response.
	nsp2N	Processing of the polyprotein pp1a and suppression of innate immune response.
	nsp3	DMV formation and RTC assembly.
	nsp4	Main PRRSV serine proteinase (cleavage of nsp3-nsp12). Inhibition of type I IFN production. Induction of apoptosis.
	nsp5	DMV formation and RTC assembly. Inhibition of type I IFN signalling.
	nsp6	Unknown function. Possible inhibition of type I IFN response.
	nsp7α	Interaction with nsp9 to facilitate viral transcription and protein translation. Inhibition of type I IFN production and signalling.
	nps7β	Induction of non-neutralizing antibody response.
	nsp8	Unknown function.
ORF1b	nsp9	RNA-dependent RNA polymerase and nidovirus RdRp-associated nucleotidyltransferase (NiRAN). Virus transcription and replication.
	nsp10	RNA helicase/ATPase. Virus transcription and replication. Induction of apoptosis.
	nsp11	Unique nidovirus-specific uridylate-specific endonuclease (NendoU). Virus RNA processing. Inhibition of type I IFN production and signalling.
	nsp12	Potentially involved in virus replication. Inhibition of type I IFN signalling.

1.4.5.1. Nsp1.

Nsp1 plays a role in both viral replication and immune evasion, and its main functions are the processing of the polyprotein pp1a, induction of sgmRNA synthesis, and downregulation of type I interferons (IFN).

Nsp1 comprises two subunits, the conserved nsp1α and the variable nsp1β. Nsp1α is distributed in both nucleus and cytoplasm, whereas nsp1β is primarily concentrated in the nuclear and perinuclear regions[142–144]. Nsp1α can be divided into three domains, namely N-terminal zinc finger motif (ZF) 1 (ZF1), the papain-like cysteine protease (PCP or PLP) 1 α (PCP1α or PLP1α) with a ZF2, and a C-terminal extension (CTE) region[145,146].

On the other hand, nsp1β contains four domains: the N-terminal domain (NTD) with metal-dependent nuclease activity, the linker domain (LKD), the PCP1β or PLP1β, and a CTE domain[147]. Both PCP1 are required for the internal cleaving of nsp1α and β from the polyprotein at the nsp1α/nsp1β and nsp1β/nsp2 cleavage sites, respectively[148,149]. Once they have been cleaved, the PCP becomes inactive through the binding with the CTE[146,147].

The ZF1 is not necessary for replication, but essential for transcription, specifically for the viral sgmRNA synthesis. It binds to nsp9 and acts as a transcription factor that induces the transition of the viral polymerase from synthesizing viral genomic RNA to sgmRNA[148,150]. Together with the other domains of nsp1, it also regulates the levels of minus-strand templates. The formation of double stranded plus/minus mRNA and its accumulation regulates viral production[151].

The role of nsp1β in the replication process is not clear. It has been described that nsp1β may be involved in the activation of the production of two proteins, nsp2TF and nsp2N, that result from a programmed ribosomal frameshifting (PRF) in the nsp2 region[152]. Moreover, its NTD nuclease domain has activity on single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), which is suggested to affect host cell nucleic acids and play a role in PRRSV virulence[147].

Nsp1 induces a high, early, and long-lasting antibody response[153,154]. Two linear B-cell epitopes have been identified in nsp1α and a discontinuous one in nsp1β[155], as well as various T-cell epitopes[156,157]. However, the precise role of these epitopes in protection is not known.

Furthermore, nsp1 proteins are also involved in host immune evasion. As mentioned previously, early studies already showed that PRRSV inhibited type I IFN[158]. Both subunits of nsp1 have been suggested to be implicated in that inhibition, probably through the disruption of dsRNA signalling pathways[142,159,160].

Several studies showed that nsp1α, and particularly its ZF1, modulates the synthesis of IFN-β via the retinoic acid inducible gene-I (RIG-I) signal pathway. This inhibition is the result of supressing NF-κB translocation and activation by different mechanisms[143,159,161,162]. Additionally, nsp1α has also been shown to inhibit the apoptosis in the early stage of infection to increase replication[163], to downregulate the surface

expression of swine leukocyte antigen class I (SLA-I)[164], to inhibit RNA silencing[165], and to be involved in pro-inflammatory responses[166], among others.

Nsp1β's immune regulation mechanisms include both the inhibition of IFN-β production and its signalling. Inhibition of IFN-β production is achieved through the inhibition of dsRNA-mediated interferon regulatory factor (IRF) 3 phosphorylation and nuclear translocation, and the inhibition of NF-κB-dependent gene induction by dsRNA[142,159,167]. On the other hand, nsp1β interferes with the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway by blocking the interferon-stimulated gene factor (ISGF) 3 nuclear translocation[142,168,169].

Besides the inhibition of type I IFN, nsp1β is also involved in other mechanisms of immune response regulation. For example, the whole nsp1β and PCP1α are involved in TNF-α suppression, by suppressing functional activities of different regions of its promoter[170,171]. Nsp1β also interacts with host proteins to regulate viral replication and RNA transcription[172], and, together with nsp11, mediates the degradation of antiviral proteins of the innate immune response[173,174], among others.

1.4.5.2. Nsp2.

Nsp2 is the largest and most variable protein among all the nsps. It is involved in proteolytic processing of the polyproteins, viral replication, and inhibition of the host's immune response.

Nsp2 is an integral membrane protein that exists as different isoforms and can be incorporated in or on the virions[175–177]. It comprises four functional regions: a N-terminal PLP2, a central hypervariable region, a hydrophobic transmembrane region (TM), and a conserved C-terminal tail rich in cysteines[43,131,178,179].

The PLP2, highly conserved among arteriviruses, cleaves the junction between nsp2 and nsp3 and, together with the TM and the C-terminal tail, is essential for viral replication[131,175,178,179]. The nsp2 C-terminal interacts with nsp3 and nsp5 and forms a complex that supports the formation of DMV, contributing to recruit other components of the viral RTC[43,131,180,181]. Additionally, nsp2 functions as a cofactor for the nsp4 protease to cleave the nsp4/5 site, known as the major processing pathway[130,182–184].

The central region of nsp2 is highly variable and mutations, deletions, and insertions are reported frequently[46,49,59,185,186]. Although sometimes deletions in nsp2 were claimed to be associated with virulence[59,187–189], their biological significance remains to be fully elucidated, since they have also been observed in less virulent strains[190,191].

In addition, nsp2 is related to PRRSV cell tropism, as several deletions have been shown to alter it[192]. Nsp2 has also been described to interact with numerous viral and cellular proteins[112,193]. For instance, in MARC-145 cells, it forms a complex with vimentin and N protein, which potentially is essential for viral attachment and replication[112].

Moreover, nsp2 interferes with the development of the immune response to the virus. The PLP2 domain belongs to the ovarian tumour domain (OTU) family of deubiquitinating enzymes. It inhibits the host immune response in different ways including the suppression of IFN-β production, by interfering with nuclear factor-kB (NF-kB) signalling and by deubiquitinating RIG-1, blocking the activation of the IRF3 pathway[159,194–196]. Nevertheless, at a later stage of infection, nsp2 may activate the NF-kB pathway with the resulting pro-inflammatory response, which is suggested to be a mechanism to induce host cell apoptosis and release the mature viral particles after PRRSV has replicated[194,197]. The C-terminal domain of nsp2, together with nsp3 and nsp5, play a role in the induction of autophagy[198,199]. Nsp2 has also been described to regulate TNF-α expression[200].

Similarly to nsp1, nsp2 triggers a robust humoral antibody response. Furthermore, numerous B-cell epitopes[153,154,200–203] and T-cell epitopes have been discovered within this protein[46,156,157,204,205].

1.4.5.3. Nsp2TF and nsp2N.

Nsp2TF and nsp2N are encoded by an ORF that overlaps the nsp2 in the -2/+1 reading frame and is translated via a -2 and -1 PRF mechanism[82]. The nsp2TF is a transmembrane protein like nsp2, yet its C-terminal hydrophobic domain is different, and it has been suggested that it directs the protein to compartments of the exocytic pathway, which is used for virus assembly[82,206]. The nsp2N is a truncated variant of nsp2[82,152]. Both proteins include the PLP2 in the N-terminal, with both proteolytic and deubiquitinating activities[82]. This deubiquitinating activity also suppresses innate immune responses[207].

Moreover, nsp2TF antagonizes the degradation of GP5 and M to promote the formation of GP5-M dimers, necessary for the viral assembly[206]. Nsp2TF can downregulate the expression of SLA-I [208]. A T-cell epitope with inhibitory effects on IFN-γ production has been identified in nsp2TF [205].

1.4.5.4. Nsp3.

Nsp3 is a transmembrane protein, with a N-terminal domain, four predicted transmembrane domains, and a cytosolic C-terminal domain[209]. This nsp is involved in the remodelling of intracellular membranes and plays a role in the formation of DMV favouring the assembly of RTC[131,180,181,209]. Moreover, nsp3 is implicated in immune evasion[210] and promoting PRRSV replication via autophagy activation[198,211].

1.4.5.5. Nsp4.

Nsp4 is the main serine proteinase (SP) of PRRSV. Functionally, nsp4 is a 3C-like serine protease (3CLSP), responsible for the cleavage of nsp3 to nsp12[43,183,212–215]. It has three domains: I, II, and III. Domains I and II form the 3CLSP, and domain III is the C-terminal domain[74,215]. The processing of nsp3-8 follows two alternative proteolytic pathways, designated as the major and the minor pathways. When nsp2 is present, it acts as a cofactor of nsp4 and this one cleaves the nsp4/5 site, producing the nsp3-4 and nsp5-8 processing intermediates, and then cleaves the nsp7/8 junction (major pathway). In the minor pathway, nsp4 cleaves nsp5/6 and nsp6/7 in the nsp3-8 and nsp4-8 intermediates instead of nsp4/5[80,130,182–184,214]. Noteworthy, a single mutation in nsp4 resulting in decreased proteolytic activity led to a decreased replication rate[216].

Nsp4 antagonizes type I IFN production by interfering the NF-κB signalling pathway[159,217–219]. Furthermore, nsp4 antagonizes the anti-PRRSV activity of interferon-stimulated gene (ISG)[220], modulates host proteins[221], and is an apoptosis inducer[222,223]. Additionally, a B-cell epitope and T-cell epitopes have been identified in nsp4[157,201].

1.4.5.6. Nsp5.

Nsp5 is a hydrophobic transmembrane protein and is involved in membrane modification and assembly of virus RTC[131,181].

Nsp5 also plays a role in PRRSV interference with the innate immunity. The C-terminal domain of Nsp5 antagonizes JAK/STAT3 signalling by inducing de degradation of STAT3, which results in the inhibition of a wide range of cytokines and growth factors[224,225]. Additionally, it has been identified to be an inducer of autophagosome formation, promoting virus replication[211]. T-cell epitopes have been identified in nsp5[157,204,226].

1.4.5.7. Nsp6.

Nsp6 is a very small peptide, poorly immunogenic, and its function remains to be elucidated[144]. It has been recently related to the suppression of NF-κB signals and inhibition of pro-inflammatory cytokine expression[221].

1.4.5.8. Nsp7.

Nsp7 is highly conserved in PRRSV but probably exists only as a transient intermediate since it is cleaved into nsp7α and nsp7β[214]. Nsp7α has been the sole form detected in virus infected cells, suggesting that nsp7β undergoes rapid degradation[144,214,227].

Nsp7 has been shown to play a critical role in facilitating both viral RNA synthesis and protein translation. On one hand, mutations and deletions in nsp7 and both subunits have proven to be detrimental to PRRSV viability and recovery[228]. On the other hand, nsp7α interacts with the RdRp structure of nsp9, potentially aiding in the formation of the RTC or assisting nsp9 in RNA synthesis[227,229].

A B-cell epitope has been identified in nsp7β that elicits high long-lasting antibody levels[154,230]. Furthermore, nsp7 is also involved in the evasion of the innate immune response by inhibiting IFN-I responses, improving the stability of the viral RNA, and modulating host proteins[221,231,232].

1.4.5.9. Nsp8.

At present, functions of nsp8 remain unknown. One T-cell epitope has been described in it[157].

1.4.5.10. Nsp9.

Nsp9 is the RNA-dependent RNA polymerase (RdRp) of PRRSV, the core component of the RTC and the responsible for the replication of the viral RNA genome[42,81,233]. The C-terminal domain of nsp9 contains the RdRp, while the N-terminal domain has the nidovirus RdRp-associated nucleotidyltransferase (NiRAN)[81,234]. Nsp9 is highly conserved among strains[235].

The RdRp domain has six conserved motifs, namely A to F, of which A and C are important for RNA polymerase activity and RNA synthesis[234,236]. The arteriviral RdRp lacks proofreading abilities[237]. NiRAN is essential for viral replication, and has been suggested to be involved in RNA ligation, protein-primed RNA synthesis, and mRNA capping[234].

Nsp9 together with nsp10 are involved in the replication efficiency of PRRSV both *in* vitro and *in vivo*, and contribute to the heightened virulence of the Chinese HP-PRRSV[238–240]. In addition, nsp9 has been shown to interact with numerous viral and cellular factors to regulate viral RNA synthesis, including PRRSV nucleoprotein and various nsps as well as several host proteins[229,241,242]. Moreover, nsp9 has been described to induce autophagosome formation[211]. Nsp9 is also related to the induction of IFN-γ and IL-10 responses, and several T-cell epitopes have been identified[156,157,205,243,244].

1.4.5.11. Nsp10.

Nsp10 is the helicase of PRRSV and, together with nsp9, is a core enzyme in the RTC[81,245]. The helicase belongs to the helicase SF1B Upf1-like superfamily and exhibits both ATPase and helicase functions, allowing for the unwinding of dsDNA/dsRNA in a 5' to 3' polarity[246–248].

Structurally, nsp10 consists of four domains: an N-terminal zinc binding domain (ZBD), a linker or intermediate 1B domain, the helicase core (domains 1A and 1B), and the C-terminal domain (CTD)[248–250]. The ZBD modulates the helicase function, is indispensable for viral RNA synthesis, is involved in the folding of nsp10, and mediates interactions with RNA molecules[42,81,246,247,249,250]. The linker 1B domain is also involved in the ATPase and helicase activities and sgmRNA synthesis[249,251]. The CTD contributes to maintaining the structural stability of the helicase and is crucial for its function[248,250].

As stated earlier, nsp10, in conjunction with nsp9, seem to govern the virus's replication efficiency contributing to the elevated virulence observed in the Chinese HP-PRRSV[238–240]. Nsp10 also interacts with both viral and cellular proteins involved in viral replication, including those RTC anchoring nsps[252], and nsp12, which has been shown to be essential for the synthesis of sgmRNA[253]. Additionally, nsp10 has been described to be an apoptosis inducer[223]. Two T-cell epitopes[243], which were related to a strong induction of IFN-γ have been identified in nsp10.

1.4.5.12. Nsp11.

Nsp11 contains the unique nidovirus-specific uridylate-specific endonuclease (NendoU) within its catalytic CTD. NendoU is a highly conserved endoribonuclease domain and a genetic marker of nidoviruses. It contains two subdomains, A and B. Subdomain A has the nuclease activity; while B maintains the overall structural conformation[254–257].

The endoribonuclease activity of nsp11 is important for both viral replication and the host's innate immune modulation. NendoU cleaves both single- and double-stranded RNA substrates 3' of pyrimidines, yet its integration into the RTC and its localization probably avoids its access to viral RNA and its degradation[81,252,255]. Viruses harbouring mutations in the active site of NendoU remain viable, but sgmRNA synthesis is impaired and the production of infectious virions is remarkably reduced[254,255,258].

Concerning the immune response, NendoU suppresses type I IFN production[159,217,256,258,259]. In addition to its endoribonuclease activity, it also possesses a deubiquitinase (DUB) activity, which also inhibits IFN-β production by inhibiting the NF-

κB activation and recruiting ovarian tumour domain deubiquitinase (OTULIN)[260,261]. Other mechanisms of inhibition of the innate immune response have been described[262,263].

Moreover, NendoU interacts with several host factors to promote PRRSV replication[174,221] and has been implicated in the inhibition of TNF-α and IL-1β production, modulation of cell cycle progression, and suppression of RNA silencing[165,171,264,265].

1.4.5.13. Nsp12.

The detailed biological function of nsp12 remains elusive; however, it potentially plays an essential role in PRRSV replication[266]. Nsp12 is a membrane-associated protein and seems to be part of the RTC, functioning as a central component for various nsps[229,252,253]. Nsp12 seems to recruit nsp11 to the RTC[252], and its interaction with nsp10 is required in viral sgmRNA synthesis[253,267].

Concerning the immune response, nsp12 inhibits ISG expression[232], and induces STAT1 phosphorylation and the expression of IL-1β, IL-8, and other cytokines and chemokines[268]. It also interacts with multiple cellular factors, mainly nucleic acid binding proteins or chaperones, to protect itself and other factors from degradation and promote viral replication[269,270].

1.4.6. Structural proteins.

The structural proteins form the physical structure of the virus particle. They are involved in the formation of the viral capsid, in the attachment and entry of the virus in the host cell, and in the budding of new viral particles. In PRRSV, structural proteins include GP2, E, GP3, GP4, GP5, ORF5a protein, M and N. Although there are reports showing the presence of nsp2 in the viral particle[176,177], it is generally considered one of the non-structural proteins. GP5, M, and N are considered the major structural proteins, while GP2, GP3, GP4, E, and ORF5a protein are classified as the minor structural proteins.

1.4.6.1. The major structural proteins.

As stated above, GP5, M, and N are considered the major structural proteins. N is the protein forming the nucleocapsid, while GP5 and M form a heterodimer by means of a

disulphide link and is the major component of the viral envelope[138,271–273]. This GP5/M complex is essential for virus assembly, budding, and subsequent particle release[138,272]. Moreover, GP5/M has been shown to be required for the structure of conformational epitopes and facilitate the cleavage of the signal peptide in certain GP5 variants[273–275]. Beyond its primary function in assembly and structure, it is also involved in the attachment to macrophages, through interaction with the host cell's HS and sialoadhesin receptor[105,108,271].

1.4.6.1.1. GP5.

GP5 is a transmembrane protein encoded by the viral ORF5[273,276,277]. The protein comprises a N-terminal cleavage signal peptide, an ectodomain with two hypervariable regions and a short highly conserved domain, one to three hydrophobic transmembrane regions, and a C-terminal intracellular hydrophilic domain[139,274,278,279].

GP5 is essential for virus formation and participates in the attachment to the cell surface by interacting with porcine sialoadhesin, through sialic acid residues present in the viral protein[108]. Nevertheless, this interaction between GP5 and porcine sialoadhesin is not essential for infectivity, but it contributes to enhance the number of viral particles on the cell surface[138,280].

For many years it was considered that GP5 contained the major neutralization epitope together with a decoy epitope close to it, which would explain the delayed neutralizing antibody responses during PRRSV infection[275,281–286]. Notably, antibodies targeting PRRSV-1 GP5 have not been shown to reduce viral infectivity, while there have been conflicting results regarding PRRSV-2[281,282,287–292]. Since accumulating evidence suggests that neutralizing antibodies are also produced against other envelope proteins, probably the key neutralization epitope is not in GP5; however, anti-GP5 neutralizing antibodies may still contribute to protection[291,293].

GP5 presents several N-glycosylation sites, of which two are conserved in both PRRSV-1 (positions 46 and 53) and PRRSV-2 (positions 44 and 51), and several others are only present in some strains[280,282,290,294]. N46 and N44 have been reported to be essential for virus particle formation and infectivity, as its N-glycan binds to porcine sialoadhesin. Additionally, N53 or N51, and the variable sites in PRRSV-2, have been suggested to help

the virus to escape from the neutralizing antibody response, by potentially hindering the neutralizing epitope recognition[108,280,285,290,294,295].

Besides the neutralizing epitope, other B-cell epitopes have been described both in the N- and C-terminal domains[202,296,297]. Immunodominant T-cell epitopes have also been identified in GP5[156,205,298,299].

Further, GP5 has also been reported to participate in the antibody-dependent enhancement (ADE) phenomenon[289] and in the apoptosis induced by PRRSV[300,301], although there are conflicting results regarding this latter[222,302,303]. Mutations and deletions in ORF5 have been described in some HP-PRRSV strains and have been suggested to be potential virulence factors[188], although this is still under debate.

1.4.6.1.2. M protein.

The M protein functions as the viral matrix membrane protein and is the most conserved structural protein[273,279]. Its structural composition includes a short ectodomain with no N-terminal signal sequence, three successive hydrophobic transmembrane domains, and a long cytoplasmic C-terminal domain[139,278]. The cysteine residue at position 8 is essential for the disulphide linkage with GP5[139,271]. Concerning the GP5/M complex and viral attachment, the M protein binds to host cell's heparan sulphate[105].

B-cell epitopes [202,289,291,292,297,304], and T-cell epitopes [156,205,305] have also been identified in M. Actually, Bautista et al.[306] showed that M protein is one of the immunodominant viral structural proteins in T-cell response.

The M protein has been described to be key target for neutralizing antibodies in PRRSV-2[288]. The deletion of an amino acid within its ectodomain has been related to a PRRSV escape mutant resistant to broadly neutralizing antibodies, possibly related to the interaction with GP5[307].

1.4.6.1.3. N protein.

The N protein, which is the nucleocapsid protein of PRRSV, is the most abundant structural protein[279,308]. It comprises an N-terminal RNA-binding domain, without a signal peptide, and a C-terminal dimerization domain[74,273,309].

N protein is responsible for forming the viral capsid, polymerising with itself through both covalent and non-covalent bonds. N protein localizes in both the cytoplasm and the nucleus and nucleolus of the host cell[308,310,311]. The nuclear localization of N is dispensable for PRRSV replication, although it seems to be involved in viral attenuation and pathogenesis[312,313].

Moreover, the N protein is phosphorylated, which possibly modulates nucleic acid binding and protein interactions. Mutation of the phosphorylation sites has been shown to affect viral replication[135,279]. N has been also reported to facilitate and regulate viral RNA synthesis[314], as well as interacting with several other host cellular factors involved in translation and post-transcriptional modifications[315,316].

Furthermore, N is highly immunogenic and induces the production of early and long-lasting non-neutralizing antibodies. Several B and T-cell epitopes have been identified[202,299,317–319]. Some of these epitopes are shared by PRRSV-1 and PRRSV-2 and this characteristic allows that N protein can be used as a single antigen for the serologic diagnosis of PRRSV-1 and PRRSV-2 infections

N has also been reported to inhibit IFN type I production[232,320], being involved in NF-kB and AP-1 activation at late infection[321], and inducing regulatory T-cells (Treg)[322], among other. N has been also involved in the ADE phenomenon[289].

1.4.6.2 The minor structural proteins.

GP2, GP3, GP4, E, and ORF5a proteins are considered the minor envelope proteins of PRRSV. GP2, GP3, and GP4 assemble into a heterotrimer complex known as the minor glycoprotein complex, which is held together by disulphide bonds. Within this structure, GP3 specifically binds to the GP2/GP4 complex. The E protein covalently interacts with the GP2-GP3-GP4 complex, which is a critical step for the complex's transportation and subsequent incorporation into virions[93,138].

This heteromultimeric complex interacts with the CD163 receptor on the host cell, facilitating the release of the viral genome. Specifically, both GP2 and GP4 establish connections with the CD163 receptor on the host cell[93]. Disruption in the expression of any of these proteins hinders cell entry, rendering virions non-infective. Thus, this complex is

crucial for PRRSV cell entry and infectivity, while it does not affect particle assembly[138]. Additionally, the minor glycoprotein complex plays a crucial role in determining PRRSV cell tropism[83,271,323].

1.4.6.2.1. GP2.

As explained above, GP2 forms a trimer together with GP3 and GP4 that is indispensable for infectivity of PRRSV. It comprises an N-terminal signal peptide, an extensive ectodomain with N-glycosylation sites, and a C-terminal membrane anchor domain with a transmembrane segment and a short cytoplasmic tail[43]).

Several B-cell epitopes have been identified in this protein, including two with neutralizing properties[202,286,291,297]. Two highly conserved N-glycosylation sites are found; however, these are not considered essential for virus particle formation or infectivity in PRRSV-1, whereas controversy arises regarding PRRSV-2[280,324,325]. In contrast to GP5, glycosylation of GP2 and the other minor glycoproteins seems not to be involved in protection from antibody neutralization[324,325].

Beyond its primary functions, GP2 has been implicated in the development of cell mediated immunity[306] and in the induction of apoptosis[326]. Furthermore, it has been shown to involved in the adaptation of PRRSV in CL2621 and MARC-145 cells[327,328].

1.4.6.2.2. GP3.

GP3 is a highly variable envelope protein that is essential for PRRSV infectivity[273,277]. Structurally, GP3 contains an N-terminal signal peptide, an ectodomain with several N-glycosylation sites, a hydrophobic region, and a hydrophilic CTD. The CTD overlaps with ORF4, and it was suggested that this region may undergo selective evolutionary pressures[201,329–331]. Besides, there is ongoing debate regarding if the protein is anchored to the membrane through the CTD alone or exhibits a hairpin-like structure[43,139,332].

GP3 induces both cellular and humoral immunity, including a neutralizing antibody response[201,202,205,289,291,297,304,333]. Six highly conserved potential N-glycosylation sites have been identified. Like GP2, there are controversial results regarding their importance for virus infectivity and immune response escape[295,324,325].

1.3.6.2.3. GP4.

GP4 is also a viral attachment protein that is essential for virus infectivity. GP4 contains a N-terminal signal sequence, an ectodomain with the CD163-binding region and a hypervariable region, and a C-terminal hydrophobic transmembrane domain, without a cytosolic tail and being GPI-anchored[273,334].

GP4 acts as a key component in the complex formation, facilitating the interaction between major and minor structural proteins on the envelope. Alongside GP2, GP4 interacts through its N-terminal with CD163 for virus uncoating and entry into the host cell[93,273].

PRRSV-1 triggers a robust neutralizing response against GP4, which diminishes the replication of homologous but not heterologous variants. The neutralizing epitope is located within the hypervariable region of this protein. Evidence indicates that it undergoes antibody-mediated selective pressure, resulting in the emergence of neutralization-resistant variants, and may function as a decoy epitope[201,291,293,333,335–337]. Moreover, mutations in the neutralizing epitope of GP4 may entail corresponding mutations in the C-terminal of GP3[201,329–331,336].

Four potential glycosylation sites have been identified. While individual mutations at these sites are not essential for PRRSV recovery, multiples mutations proved to be lethal[324]. T-cell epitopes have also been identified in GP4[205,244,299].

1.4.6.2.4. E protein.

E protein is highly conserved and is translated from an alternative reading frame in ORF2. E protein is integrated into the viral envelope and comprises a N-terminal with a conserved myristoylation motif, a hydrophobic transmembrane region, and a conserved hydrophilic C-terminus[338–340]. The myristoylation of the N-terminal is suggested to enhance viral replication by promoting virus entry[341].

Protein E is covalently linked with the GP2/GP3/GP4 heterotrimers[138]. It also interacts non-covalently with N, possibly contributing to the stabilization of the virion core structure, once N associates with RNA[128,279].

Although not critical for virus particle assembly, E protein is crucial for infectivity by facilitating virus uncoating and genome release in the cytoplasm. Deletion of protein E impairs viral particle production, rendering them non-infectious[128,138].

Beyond its role in cell entry, the ion activity of protein E disrupts ion homeostasis, subsequently activating PAM inflammasome and resulting in the production of IL-β[342]. Additionally, it regulates the immune response and promotes viral replication through several mechanisms[210,343,344], and induces apoptosis[345]. PRRSV-infected pigs develop specific antibodies against E protein[339]. Furthermore, E protein along with other proteins has been related to the attenuation of HP-PRRSV strains after continuous passages[346].

1.4.6.2.5. ORF5a protein.

ORF5a, is a minor non-glycosylated hydrophobic structural protein encoded by an alternative reading frame of ORF5 subgenomic RNA. It comprises a N-terminal ectodomain, a hydrophobic transmembrane domain, and a long C-terminal domain[347,348].

ORF5a interacts with itself non-covalently and with GP4 and E proteins[349]. ORF5a is essential for virus viability and replication[139,347,350], and it is suggested to be involved in RNA-binding[348].

In addition, the ORF5a protein induces a non-neutralizing antibody response[348,351], and has been reported to be involved in promoting the inflammatory response[343].

1.5. PRRS, THE DISEASE

PRRSV primarily targets mature $CD163^+$ macrophages[83] and thus can be found in most lymphoid tissues. Despite the virus does not replicate in the epithelial cells of the airways or the cells of the placenta other than macrophages, the infection commonly presents as a respiratory or reproductive disease. A crucial aspect of this infection is that animals, particularly young ones, can remain infected for an extended period without exhibiting clinical signs, thereby contributing to the spread of the disease.

In the respiratory tract of the pig, PRRSV-susceptible macrophages are scarce in the upper or middle airways; in contrast, the alveolar wall is rich in $CD163^+$-macrophages[84,352,353], usually named as porcine alveolar macrophages (PAM). The pig lung

is also rich in the so-called pulmonary intravascular macrophages (PIM), namely, a resident population of macrophages adherent to the capillary endothelium of lungs[354,355]. These PIM are also $CD163^{+}$ and susceptible to PRRSV[356–359]. The existence of these two populations of susceptible macrophages in lungs explains why PRRSV causes respiratory disease with a pneumonic nature.

In the pregnant sow, the systemic infection caused by PRRSV allows transplacental infection, once susceptible macrophages infiltrate the chorionic trophoblast. A combination of placentitis and foetal infection leads to the reproductive failure[360], characterized by late-term abortions, stillbirths, and weak-born piglets in pregnant sows.

1.5.1. Pathogenesis.

PRRSV infection unfolds in two phases: an acute phase with widespread viral replication causing viraemia and clinical signs, and a subsequent persistent phase marked by lower-level replication in lymphoid tissues, that can extend for months, but ultimately, the infection is resolved by the immune response.

Under natural circumstances, PRRSV enters the organism primarily through the oronasal route, although other routes like sexual, percutaneous (bites or iatrogenically), or transplacental are possible. Following this initial exposure, the infection of the macrophages in lymphoid tissues at the entry site facilitates viral dissemination into the bloodstream through lymphoid tissue drainage. This viraemia will allow the virus to infect macrophages in various organs, including lymphoid organs, lungs, and reproductive organs, as well as tissues such as the bulbourethral gland, the seminal vesicles, or the placenta[129,361–363].

Viraemia can be detected as early as six to twelve hours following infection and reaches its peak between four- and fourteen-days post-infection[361,364,365]. In this regard, the duration of the viraemia depends on the age of the infected animal. In general, the younger the animal the longer the viraemia[364,366,367]. In weaned pigs, viraemia usually lasts three to five weeks, although it has been described to persist up to three months, while lasting around one or two weeks in adult pigs[361,363–365,368–371]. Nonetheless, the duration of

viraemia is also influenced by factors such as the virus strain, the age and immune status of the animals[364,366,367,371,372].

In any case, at some point viraemia ceases, but the virus can persist in lymphoid tissues, particularly in tonsils, at a diminished rate of replication for weeks or even months[363,369,373,374]. This post-viraemic period usually presents as subclinical. Throughout this stage, the possibility of PRRSV transmission still exists, with reported contagiousness lasting up to three months after the onset of the infection[365,369,370,375,376]. Besides, infective virus has been successfully isolated from lymphoid organs up to 157 days post-infection, while viral RNA has been detected up to 251 days post-infection in experimentally infected piglets[370,373]. In most instances, replication in lymphoid organs gradually decreases over time, eventually clearing from the animal around three to four months post-infection[369,370]. Nonetheless, some animals may experience a recurrence of viraemia and shedding in response to stressful conditions[377].

The mechanisms involved in virus persistence have not been fully described, yet potentially the host's immune response and the selection of viral subpopulations during the infection may play a significant role[378–380].

Clinical signs and lesions during PRRSV infection result from various mechanisms, including death by apoptosis or necrosis of PRRSV-infected macrophages or bystander cells[381–388], release of pro-inflammatory cytokines[382,389–391], and the interference with the immune system[129,161,321,392–395]. These promote virus replication and persistence, while also increasing the susceptibility to a wide range of secondary pathogens[396–401].

In the context of transplacental infection (further reviewed[360]), PRRSV only breaches the maternal-foetal barrier and crosses the placenta during the third trimester, subsequently infecting the foetuses[58,402–407]. The absence of transplacental transmission before day 80-85 of gestation is due to the lack, until that point, of susceptible $CD163^{+}$/$CD169^{+}$ macrophages in the endometrium and the placenta, along with local factors in the development of the placenta, such as the formation of areolae[360,408,409]. Occasionally, embryo infection post-implantation may occur in early gestational infections, while infections at mid-gestation do not lead to transplacental transmission[360,404,410,411].

The way in which the virus crosses the placenta has not been elucidated yet. Foetal infection can occur either through direct crossing of free viral particles across the six layers of the placenta, cell-to-cell transmission via epithelial cells, or by migration of infected macrophages[360,409,412,413]. When PRRSV reaches the placenta, it replicates in foetal placental macrophages[387], which then reach foetal blood and organs. PRRSV may infect neighbouring siblings by migration of PRRSV-infected macrophages, or by free PRRSV particles through adherent extremities of foetal membranes or blood anastomoses between allantochorions[360]. Hence, foetuses can be infected at various time points, leading to diverse outcomes of foetal preservation and PRRSV status upon birth[414].

PRRSV replication in foetal implantation sites induces apoptosis in infected and by-stander cells, leading to the detachment and foetal death[360,387,415,416]. Within foetuses, PRRSV replicates in thymus, lungs, liver, spleen, tonsils, and lymph nodes, ultimately leading to foetal death or birth of infected piglets[402,406,417,418]. Thymic infection by highly virulent strains can lead to severe disruptions in thymic functionality and the B-cell repertoire[419–422].

Noteworthy, in utero infections can give rise to long lasting PRRSV-viraemic piglets, with reports indicating infection persistence up to 132 days of age[423,424]. Congenitally infected piglets have transmitted PRRSV to sentinel animals up to 112 days after birth[424]. Certainly, these animals may have the potential to shed PRRSV throughout their entire production life, making them potentially significant contributors to the spread of the virus on the farm.

1.5.2. Clinical signs and lesions.

The two primary clinical features of PRRS are reproductive disorders in sows and respiratory problems in weaners and growing pigs[2,18]. In any case, infections can range from subclinical to fatal and the disease's severity is related to the virulence of the strain, the immune status of the infected animal, and other circumstances (age, breed, welfare, concomitant infections, etc.)[364,396,410,425–431].

The reproductive form of PRRS (further reviewed[368432433]) usually is observed when PRRSV infects naïve sows or pregnant females with insufficient immunity against

the infecting strain. The initial phases of the infection in pregnant sows are characterized by unspecific signs, such as fever and a decrease in the feed consumption, which rarely last more than a few days[8,11,403]. The subsequent course will be determined by the stage of gestation in the affected sow. Thus, if the infection occurs in early gestation, before day 80-85, most probably the sow will recover showing no other clinical signs. In females affected after day 85-90 of gestation, the outcome of the infection will be a late-term abortion with the delivery of a mix of fresh and mummified piglets. When the infection occurs in very late gestation, advanced or delayed farrowing is possible with the birth of stillborn piglets or weak-born animals[8,11,402–404]. When a new strain enters a naïve or non-sufficiently immune herd, the result can be an outbreak. The prototypical reproductive PRRS outbreak is preceded by a decrease in the feed consumption followed by an abortion storm[11]. The reproductive outbreak may last for several months before returning to the baseline productivity[434]. During this period, increased mortality rates, respiratory distress, and secondary infections in piglets in the farrowing units are common[11]. In more endemic phases, abortions, mummified foetuses, and stillbirths are recorded more sporadically but may maintain the reproductive indexes above the intervention levels for months[425]. With very virulent isolates it is not uncommon to record sow mortality that, in some cases, can reach values above 10%[58,59,71,431,434].

The respiratory disorders are mainly manifested in weaned and growing pigs, marked by signs of respiratory distress, accompanied by fever and the associated signs (anorexia, lethargy, etc.). Occasionally, cyanosis can be observed. Altogether the infection leads to a poor growth performance and increased secondary bacterial infections [19,56,435–437].

Boars and non-pregnant sows may be asymptomatic or only exhibit temporary hyperthermia and anorexia[11,410,411,438]. PRRSV infection in boars may result in alterations in the semen quality, such as reduced sperm motility, increased morphological alterations, and alterations in acrosomes[438–441].

Following the restoration of the farm's clinical situation, most often because the establishment of some level of immunity, herds enter an endemic phase of the infection where the virus still circulates within the breeding herd. In this phase, infections of sows are commonly subclinical, and the reproductive impact is usually detected only when productive records are examined. However, clinical disease tends to appear in the

susceptible population, particularly in nursery-grower pigs, when maternally derived antibodies (MDA) fade out[425], and in replacement gilts along with their offspring [442]. The introduction of non-immune gilts may contribute to periodical rebounds of the incidence in the sows' stock[368,425,433].

Regarding the nurseries, once the infection arrives the disease tends to establish itself endemically. This is particularly evident when all-in/all-out procedures are not strictly followed. Given the extended duration of the infection in young pigs, the short-lived nature of MDA, and the persistence of vertical transmission for a time after the initial infection of the breeding stock, respiratory disease becomes recurrent in nurseries if no intervention is implemented.

The hallmark lesion of PRRSV infection in its respiratory form is interstitial pneumonia[56,368,425,426]. Microscopically, this lesion is characterized by thickening of the alveolar septa, resulting from a mixed inflammatory infiltrate primarily composed of macrophages and, to a lesser extent, lymphocytes, along with hypertrophy and hyperplasia of type II pneumocytes[56,426]. PRRSV-1 strains appear to be less pneumovirulent than PRRSV-2 ones[443], although the discovery of highly virulent PRRSV-1 isolates could alter this picture. Nonetheless, concurrent infections often complicate or obscure the identification of these lesions. In the field, it is common to find suppurative bronchopneumonia or other type of bacterial pneumonias in PRRSV-infected animals suffering complications. Enlarged lymph nodes is also a common finding in PRRSV-infected pigs, with microscopic lesions such as hypertrophy, hyperplasia, and necrosis in the germinal centre, and cystic spaces and polykaryocytes in the cortical tissue[361]. It is worth mentioning that highly virulent isolates can produce a severe depletion and atrophy of thymus in young piglets, or after intrauterine infection (further reviewed[419]).

In sows, PRRSV has been described to cause metritis, endometritis, and vasculitis[444]. In the foetuses, haemorrhages in the umbilical cord, myocarditis, arteritis, and encephalitis in foetus have been reported[444,445].

In the context of virulent PRRSV strains, the disease is marked by high morbidity and mortality rates and elevated fever (40.5-42°C), along with lethargy, anorexia, diarrhoea, petechiae, among others. Common gross lesions are severe interstitial pneumonia, thymic atrophy, lymphadenopathy, haemorrhagic lesions in lungs, lymph nodes, kidneys, and skin[59,65,67,68,70,72].

1.6. PRRSV AND THE HOST'S IMMUNE RESPONSE

PRRSV exhibits a remarkable ability to circumvent the host immune response through various mechanisms. The virus disturbs, regulates, or hinders numerous processes involved in both innate and adaptive immunity, resulting in a non-fully effective host's innate immune response, coupled with a delayed protective antibody and cell-mediated immune response (further reviewed[129,392–394,446–452]). The result of this complex interaction with the immune system is the development of a long viraemia, followed by the prolonged replication of the virus in the lymphoid tissues. By doing so, PRRSV enhances the likelihood of transmission, thereby contributing to the maintenance and spread of the virus within porcine populations. Some of those mechanisms seem to be related to the particular strain infecting the pig, underscoring the significant role of genetic diversity in these processes. Besides, the host's immune response exerts selective pressures on PRRSV, driving viral evolution and influencing its genetic diversity.

1.6.1. PRRSV's evasion strategies against the innate immune response.

Innate immunity serves as the initial line of defence against infections, recognising and responding to pathogens in a non-specific manner. Also, it is essential for the development of adaptive immunity, by creating the adequate milieu and participating in antigen presentation through antigen-presenting cells (further reviewed[453]).

One of the main mechanisms by which PRRSV is thought to subvert the innate immune response of pigs is by inhibiting or dysregulating type I IFN production. Early studies using PAM showed that exposure of these cells to PRRSV did not result in a significant production of IFN-α, while inhibiting the production of IFN-α induced by the exposure to transmissible gastroenteritis virus[158]. Although most PRRSV strains seem to have this capability, Lee et al.[454] reported that different IFN-inducing phenotypes may exist. Further studies have shown that PRRSV is susceptible to the action of IFN and both type I and type II IFN are capable of inhibiting PRRSV replication[85,455–460].

The main cell specialised in IFN production against viruses is the plasmacytoid dendritic cell (further reviewed[461]). Conflicting results have been obtained regarding PRRSV effect on these cells. Calzada-Nova et al.[462] reported the inhibitory effect of

PRRSV on the production of IFN-α. Conversely, Baumann et al.[463] documented that most of the examined PRRSV-1 and -2 strains were unable of inhibiting the production of IFN-α in plasmacytoid dendritic cells, with only very virulent strains demonstrating this capability.

The mechanisms by which PRRSV inhibits type I IFN are diverse and include all the steps from the inhibition of the expression of innate immunity receptors to the different steps in the signalling pathways (further reviewed[140,141,321,464–466]). In the following section we will review briefly the different mechanisms used by PRRSV against the innate immune response.

1.6.1.1. Inhibition of Toll-like receptor (TLR) expression.

Toll-like receptors (TLR) and retinoic acid inducible gene-I (RIG-I)-like receptors (RLR) are crucial components of the innate immune system involved in recognizing viral pathogens (Figure 6). PRRSV has been shown to regulate or be involved in the expression of endosomal TLR, such as TLR3, TLR7, and TLR9, being both up- and downregulation reported in previous studies, which has been suggested to be strain- and time-dependent[395,467–474]. PRRSV also targets RIG-I and its downstream signalling adaptor through nsp4 and nsp11[219,258,472,473,475].

1.6.1.2. Inhibition of type I IFN production pathway.

PRRSV inhibits the production of type I IFN, IFN-α and IFN-β, by hindering with the activation of various intermediate adaptors and kinases, as well as interfering with transcriptional factors, including NF-κB, IRF3, and IRF7, and other components of the cascade (Figure 6). Noteworthy is the involvement of distinct viral proteins, namely nsp1α, nsp1β, nsp2, nsp4, nsp7, nsp11, and N protein, being nsp1 the strongest IFN antagonist. As detailed *in Section 1.4.5. Functions of the non-structural proteins*, each of these proteins independently suppress type I IFN response by targeting different steps within this intricate cascade[143,159,259,462,475]. Nevertheless, as stated earlier, it seems that modulation of IFN-α could be strain-dependent[454,458,476–478].

On the other hand, PRRSV has been shown to activate NF-κB through N and nsp2 proteins during late infection, resulting in the production of cytokines and preventing cells from apoptosis, to promote viral replication and survival in the host[194,197,218,475,479,480].

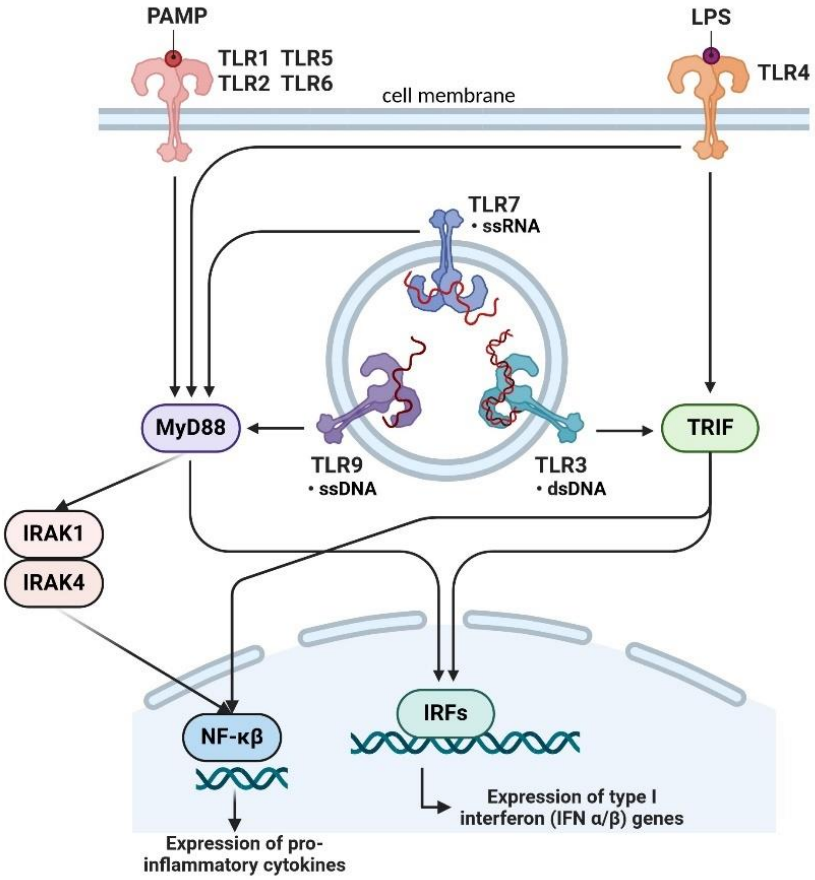

Figure 6. Induction of type I IFN expression. Several PAMPS can induce the production of type I IFN by activating Toll-like receptors (TLR) or other receptors, such as retinoic acid inducible gene-I (RIG-I)-like receptors (RLR) (not shown here). Upon binding of the ligand, the intracellular domain of the TLR activates a signalling cascade based on the MyD88 molecule, TRIF, or both. The activation of these molecules results in the phosphorylation and activation of the interferon regulatory factors (IRF). These factors, particularly the dimer formed by IRF3 and IRF7, translocate to the nucleus, where they associate to IFN-β promoter and activate the transcription of type I interferons. In parallel, activation of TLRs via MyD88 results in the activation of interleukin-1 receptor-associated kinases (IRAK). This activates the NF-κβ and its translocation to the nucleus, where it activates the transcription of pro-inflammatory cytokines. dsDNA: double-stranded DNA; IFN: Interferon; IRAK: Interleukin-1 receptors associated kinase; IRF: Interferon regulatory factor; LPS: Lipopolysaccharide; MyD: Myeloid differentiation primary response; NF-kB: nuclear factor kappa; PAMP: Pathogen-associated molecular pattern; ssDNA: single-stranded DNA; ssRNA: single-stranded RNA; TLR: Toll-like receptor; TRIF: Toll/Interleukin-1domain receptor-containing adapter protein. Reviewed in Kawai and Akira[481] and Schneider et al.[453]. Created with BioRender.com.

1.6.1.3. Inhibition of type I IFN signalling pathway.

Figure 7 represents the type I IFN signalling pathway. PRRSV not only restricts the activation of the JAK/STAT pathway by inhibiting type I IFN production but also actively interferes with JAK/STAT signalling through multiple mechanisms, resulting in the inhibition of specific ISG synthesis. Once again, multiple proteins of PRRSV, including nsp1β, nsp5, nsp7, nsp11, nsp12, GP3, and N, have been identified as key players in this interference, targeting various steps within the JAK/STAT signalling cascade[142,168,169,232,462224,225,262,263]. The interference of IFN signalling could also be strain-dependent[232,482].

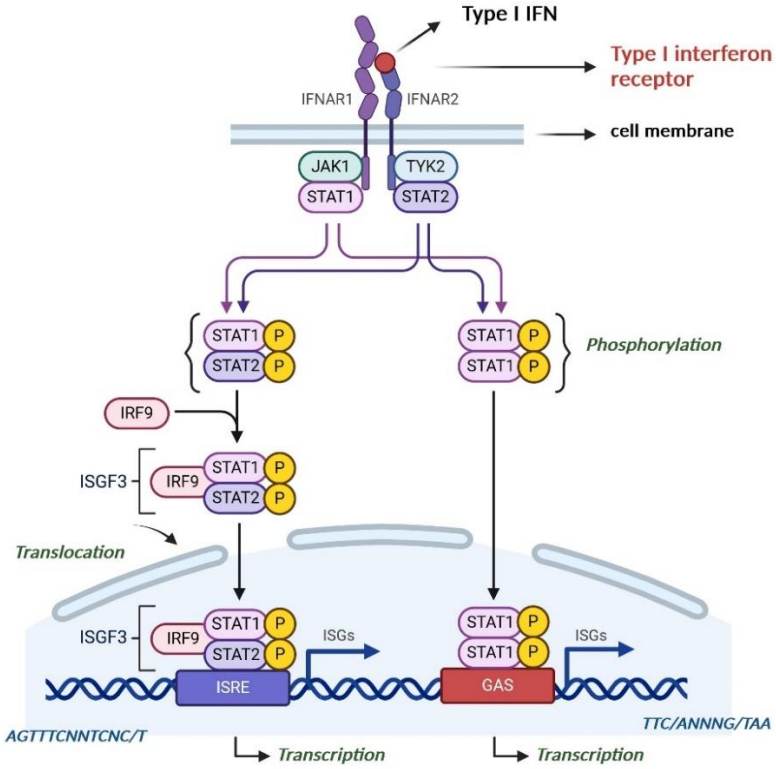

Figure 7. Canonical signalling pathway of type I interferons. The type I IFN receptor (IFNAR) consists of two subunits and is a transmembrane protein associated with two kinases (JAK1 and TYK2) and two proteins (STAT1 and 2), which act as signal transducers and activators of transcription. Binding of type I IFN to the receptor results in the phosphorylation of the STAT proteins. The phosphorylated proteins then form a complex with IRF9, known as ISGF3, which translocates to the nucleus. In the nucleus, ISGF3 binds ISRE, initiating transcription. Additionally, phosphorylated STAT 1 and 1 also translocate to the nucleus, where they will bind elements in the interferon-activated site, initiating again transcription. Consensus sequences of the interferon-stimulated elements are shown in blue. Interferon-induced transcription may involve hundreds of genes. GAS: Gamma interferon activated site; IFN: Interferon; IFNAR: Interferon-alpha/beta receptor; IRF9: Interferon regulatory factor 9; ISG: Interferon-stimulated gene; ISGF: Interferon-stimulated gene factor 3; ISRE: Interferon-stimulated response elements; P: phosphorylation; JAK: Janus-activated kinase; STAT: Signal transducer and transcription activator; TYK: Tyrosine kinase. Reviewed in Schneider et al.[453]). Created with BioRender.com

1.6.1.4. Inhibition of interferon-stimulated genes (ISG) and other antiviral proteins.

PRRSV has also been shown to modulate the expression or function of various ISGs and other antiviral proteins to interfere with the antiviral response[174,196,344,483,484].

1.6.1.5. Tumour necrosis factor alpha (TNF-α), interleukin- (IL-) 10, and other cytokines.

Besides the inhibition of type I IFN, PRRSV also regulates several key cytokines like TNF-α, IL-1, IL-6, IL-8, and IL-10, impairing the immune response to promote its survival (further reviewed[437,485]). However, the induced cytokine profile varies among PRRSV strains, animals, organs, and cell types[171,235,486–489].

TNF-α, a key pro-inflammatory cytokine, has been shown to reduce PRRSV replication[391,490]. In turn, PRRSV can hinder its production[235,390,490–493] and nsp1α, nsp1β, nsp2, and nsp11 have been associated with its downregulation, as specified in *Section 1.4.5. Functions of the non-structural proteins*[170,171,200]. In contrast, some reports have also described overexpression of TNF-α production by PRRSV [382,391,478,487,488,494].

PRRSV also modulates the regulatory cytokine IL-10. PRRSV induces IL-10 production both *in vitro* and *in vivo*, through nsp1, GP5, and N[322,493,495–503]. While IL-10 functions as a potent anti-inflammatory cytokine, dampening the inflammatory response and mitigating tissue damage, it may also prolong virus persistence by regulating the cell-mediated response. However, some strains lack the capacity to induce this cytokine, thereby it has been also suggested to depend on PRRSV strain, together to other factors such as animal and age[235,504–508].

PRRSV also modulates other cytokines such as IL-1, IL-2, IL-4, IL-6, and IL-8. In some studies, it has been shown that PRRSV limits the release of the pro-inflammatory cytokines resulting in a weak and delayed activation of the adaptive immune response, while in others an increased level of these cytokines has been reported[235,390,468,486,487,494,498,504,509]. In the lungs, high levels of pro-inflammatory cytokines seem to be linked to the development of interstitial pneumonia[389,491,501,510].

1.6.1.6. Other mechanisms of subversion of the innate immune response.

a) Inhibition of the maturation of dendritic cells (DC): Exposure to PRRSV may result in an inhibition of the maturation of DC, evidenced by the lack of an increase in the expression of maturation molecules such as SLA-I, SLA-II, CD80/86, or CCR7, as well as the absence of cytokine release, or no enhancement in antigen presentation capabilities[395]. This effect seems to be dependent on the strain. It has been reported that moderate and low virulent strains might not activate conventional DC, while highly virulent isolates may induce a strong Th1 polarization[511,512].

b) Inhibition of phagocytic and bactericidal activity of PAM: PRRSV has also been attributed to cause a lower phagocytic and bactericidal activity of PAM, increasing the susceptibility to secondary bacterial infections[356,513,514].

c) Inhibition of natural killer (NK) cells: IFN are also responsible for the activation of NK cells, which help in the clearance of virus-infected cells through their cytotoxic activity and the production of IFN-γ and TNF-α during viral infections. PRRSV infection can produce a suppression of the cytotoxic activity and function of NK cells, by reducing PRRSV-infected PAM susceptibility to NK cells, and a possible transient inhibition of their frequency[158,502,509,515–517].

d) Interference with antigen presentation and T cell activation: PRRSV has also been described to modulate the expression of the porcine major histocompatibility complex (MHC) SLA-I and SLA-II in antigen-presenting cells (APC), impairing antigen presentation and T-cell activation[85,488,506,508,518]. Several proteins, including nsp1α, nsp2TF, nsp4, and GP3, can independently downregulate the expression of SLA-I[164,208]. This mechanism also varies among different PRRSV strains[488,508,519].

1.6.1.7. Other strategies of PRRSV targeting the immune response.

Despite interacting with both the innate and adaptive immune responses of the host, PRRSV is involved in additional mechanisms such as apoptosis and micro-RNA (miRNA) regulation, among others (further reviewed[141]).

PRRSV modulates apoptosis, a programmed cell death involved also in host defence against viral infection, both *in vitro* and *in vivo*. Despite apoptosis induction and

inhibition have been both reported, there is a suggested temporal modulation of apoptosis by PRRSV throughout the course of infection. Early in infection, PRRSV may inhibit apoptosis through GP2 to enhance viral replication[326], while in later stages it induces apoptosis through GP5, E, nsp4, and nsp10, facilitating the release of viral particles[222,223,300,301]. Moreover, as previously mentioned, apoptosis occurs not only in cells directly infected by PRRSV, but also in neighbouring cells[381–388,520–522].

Another target of PRRSV is miRNA, which are involved in regulating immune responses. PRRSV has been shown to up- or downregulate certain miRNA, resulting for instance in the inhibition of type I IFN signalling pathway, or the expression of antiviral proteins, thereby promoting viral replication[523–525].

1.6.2. Adaptive immune response against PRRSV.

The mechanisms underlying protective immunity against PRRSV remain elusive. Both neutralizing antibodies (NAb) and cell-mediated immunity (CMI) contribute to protection and viral elimination. However, the acquired immunity is insufficient to fully prevent reinfection when exposed to heterologous strains. Besides, the adaptive immune response to PRRSV faces challenges. Upon infection, the humoral response displays a rapid induction of non-neutralizing antibodies but NAb develop much slower and usually at low levels (further reviewed[275]). This is coupled with a functionally impaired CMI.

1.6.2.1. Humoral immunity.

Following infection, an early and robust antibody response unfolds rapidly, with antibodies appearing between five to nine days post-infection and most animals undergoing seroconversion by day 14 post-infection[498,526–530]. Primarily, these antibodies target the N protein, and to a lesser extent to the M protein and GP5[527,531,532]. However, nsp2 is probably the immunodominant protein of PRRSV and antibodies against it tend to reach high titres later on[201,202].

As in most infections, the initial antibodies are predominantly of the immunoglobulin (Ig) M class that peak between two to three weeks post-infection, and fade around forty days post-infection. IgG reach their peak between three to four weeks

post-infection and may persist for several months[528,529,532,533]. Besides, the antibody response kinetics may also be strain-dependent, probably related to the strength of viral replication[364,372,534]. In addition, antibodies directed to other PRRSV proteins are also produced, including nsp1 and nsp7, which are highly immunogenic and have a similar pattern as that of the N protein, yet with an extended duration[153,154,372,533].

The initial antibodies are non-neutralizing and do not correlate with protection[529,534–537]. Their potential role in antibody-dependent cell-mediated cytotoxicity is unclear since, apparently, infected macrophages are resistant to antibody-dependent lysis mediated by complement[538]. However, they probably contribute to the ADE phenomenon of PRRSV replication, boosting viral infectivity and possibly increasing PRRS severity and susceptibility when low antibody levels are present[539–542]. PRRSV ADE may play a role in the suppression of the host's innate immune response downregulating type I, II, and III interferons[543–545]. However, the occurrence and importance of ADE in PRRSV infection remain somewhat controversial[546,547].

Neutralizing antibodies (NAb) appear late in infection, after two to four weeks post-infection and at low levels (below 1:32-1:64, and usually between 1:2 and 1:16)[337,498,529–532,535,548]. Noteworthy, induction and titres of NAb are both strain- and animal-dependent, with some strains being very poor inducers of NAb and certain individuals not developing NAb responses at all [498,527,528,531,532,549–551].

As detailed is *Section 1.4.6. Structural proteins*, neutralizing epitopes have been mapped in GP2, GP3, GP4, GP5, and M protein [282,289,291,293,304,333,336,337,552,553]. Early studies located the main neutralizing epitope on GP5[281,282,287,288,290,531,536,541,553]. However, antibody titres against GP5 do not appear to align with NAb titres, and uncertainties exist regarding its importance in *in vivo* neutralization of the virus. At present, anti-GP5 NAb are not considered the main type of neutralizing antibodies against PRRSV[291,292,533]. Because of the function of GP5 in the attachment and internalization of PRRSV[108], these GP5 antibodies probably block interaction with porcine sialoadhesin.

As explained before, CD163 is the essential receptor for PRRSV. This interaction takes place with the heterotrimer formed by GP2, GP3, and GP4, and thereby most probably the main neutralization epitopes are located in these proteins[83,93,291,293,295,324,337]. The neutralization epitope in GP4 has been well described[293,335–337]. It is located in a

variable segment of that protein and can act as a diversion epitope for the immune system[293,336]. Neutralization epitopes in GP2 or GP3 have not been identified yet. Some authors also suggested that nsp2 may play a role in the development of neutralizing antibodies[554].

The precise role of NAb in protection against PRRSV infection is unclear. *In vitro* studies have demonstrated the ability of NAb to block PRRSV infection in PAM[555]. Moreover, the passive transfer of NAb titres at a level of 1:16 proved to be protective for pregnant sows, preventing reproductive failure and transplacental infection[556]. Similarly, NAb titres at levels of 1:8 and 1:32 conferred protection upon piglets, guarding them against viraemia (>1:8) and providing sterilizing immunity (>1:32), respectively[537]. Additionally, exposure to PRRSV or vaccination provides a certain level of protection against future exposures, at least to the homologous strain, correlating with the development of NAb, where higher titres are linked to enhanced protection[287,407,534,541,557–560]. Nonetheless, attaining protective NAb titres usually requires repeated exposures or multiple vaccinations, especially with different strains as it tends to result in higher NAb levels[371,557,561–564].

In contrast, PRRSV can persist for weeks post-infection in lymphoid organs with high titres of homologous circulating NAb[373]. Moreover, experimental findings suggest that viraemia and viral replication can persist even in the presence of NAb, while it may also resolve with no detectable levels of NAb[295,371,498,505,529,533,562,565,566]. However, viraemia may clear with the development of NAb[541], sparking debates about the role of humoral immunity in clearing natural PRRSV infections. Therefore, while adequate levels of NAb prior to infection may offer protection against the disease and may be a possible correlate of protection for PRRSV, they seem to contribute only partially to virus clearance.

Notably, cross-neutralization is rare between PRRSV strains, even among closely related strains, resulting in inconsistent and partial protection against heterologous strains[337,515,534,551,555,561,567–569]. However, certain animals produce broadly NAb able to cross-neutralize heterologous strains[307,546,547,551,570]. While the induction of broadly NAb may be also strain-dependent, immunization with different PRRSV strains or repeated exposure to the same strain may also lead to a broader cross-protection NAb response against different strains[534,546,547,551,570]. This broadly neutralizing activity might be related

with M protein[307]. Moreover, these broadly NAb seem to confer protection against heterologous challenge[546,547].

The delayed development of NAb and their low titres have been attributed to factors such as a poor cytokine stimulation and impairment of antigen presentation[499,518,571], N-glycosylations shielding neutralizing epitopes[295,572], decoy epitopes[281–285], and genetic variability. Noteworthy, changing glycosylation patterns in GP5, including losses and acquisitions, have been observed in PRRSV strains, influencing both the production of NAb in infected pigs and the susceptibility of PRRSV to NAb[282,290,294,295,371,573]. For instance, the observed glycosylation changes of GP5 in Spanish PRRSV strains from 1991 to 2005 aligned with the selection of strains that elicit weaker NAb responses[573]. Conversely, glycosylation of the minor glycoproteins seems not to be involved in neutralizing antibody response escape[295,324,325].

For PRRSV-2, GP5 contains a principal neutralizing epitope along with an additional highly immunodominant epitope, proposed to act as a decoy epitope. This decoy epitope is hypervariable and induces a strong and early non-neutralizing antibody response, which is suggested to block the exposure of the neutralizing epitope, delaying the NAb response[281–285].

1.6.2.2. Cell-mediated immunity.

Cell-mediated immunity (CMI) has been shown to correlate with the recovery from infection and protection against transplacental transmission. Once more, the induction of CMI seems to be strain- and animal-dependent[371,478,505,508,574,575].

Lymphoproliferation was primarily used to explore the cell-mediated response against PRRSV[576]. The onset of the proliferative response of lymphocytes is detectable at four weeks post-infection, peaking at seven weeks post-infection, and decreasing eleven weeks post-infection, with CD4 T cells being the main proliferating subset[576,577]. M and N proteins seem to be the main inducers of the proliferative response, although GP5, GP2, and GP3 have also been involved[576,578].

CMI can be also assessed by determining the frequencies of PRRSV-specific IFN-γ-secreting cells using techniques like IFN-γ enzyme-linked immunosorbent spot

(ELISPOT) or intracellular flow cytometry. The appearance of PRRSV-specific IFN-γ-secreting cells is detectable at two to three weeks post-infection, followed by a slow, fluctuating, and erratic development over the subsequent weeks, until reaching a plateau[367,498,505,530,565,566]. It is worth noting that the frequencies of PRRSV-specific IFN-γ-secreting cells are comparatively lower compared to those induced by other viral agents of pigs[530,565]. Analysis of the cells involved in the IFN-γ response revealed a predominant presence of CD4/CD8 double-positive T cells, with a lesser contribution of CD8αβ$^+$ single-positive T cells[565]. M protein, N protein, and GP4 seem to be the stronger inducers of IFN-γ[299,579].

Elevated levels of IFN-γ-secreting cells appear to contribute to protection against viraemia, reproductive disorders, and respiratory disease[505,562,565,575,580–582]. However, conflicting findings on protective correlates have been reported[534,583]. Regardless, the delayed viral clearance and the ability of PRRSV to persist in the host suggest that the CMI lacks sufficient strength to clear rapidly the infection[566,575,583].

Studies indicate that CD8 cytotoxic T lymphocyte (CTL) do not develop during PRRSV infection with the rapidness and strength observed in other viral diseases[584,585]. Instead, CD4 CTL seem to be involved in memory responses against PRRSV[577], and elevated levels of these after vaccination seem to contribute to protection[586]. In fact, cytotoxic T lymphocyte responses by systemic CD4 CTL have been shown to be correlated with the protection against viraemia in PRRSV-2 and transplacental transmission in PRRSV-1[586–588]. CD8 T cells seem to predominate in lungs and may be involved in virus clearance in tissues[587,589]. Also, γδ T cells release IFN-γ in PRRSV-infected animals and may play important roles in lymphoid tissues[587,590].

Several T-cell epitopes have been mapped among PRRSV proteins (GP2, E, GP3, GP4, GP5, M, N, nsp1, nsp2, nsp5, nsp9, nsp10, and nsp11)[204,205,243,244,298,299,305,306], but the precise correlation with protection is not known.

The role of Treg in PRRSV infection has been a subject of much debate for years. Several studies showed that PRRSV-2 induces proliferation and activation of Treg; however, this could not be proved for PRRSV-1[507,508,591,592]. The N protein has been involved in Treg induction[322,593].

1.6.3. Maternally-derived immunity.

Similar to other diseases, the role of maternally-derived immunity in PRRSV infection is two-edged. Passive antibodies, transferred with colostrum, remain detectable for a period of time that rarely surpasses the sixth week of age[594]. These antibodies mirror the sow's immunity and may or may not contain NAb. Their duration correlates with the titres of sows[594].

On one hand, current evidence indicates that maternally-derived immunity can partially protect piglets from infection. On the other hand, these antibodies may interfere with the development of active immunity following vaccination. For example, the transfer of passive antibodies to piglets conferred protection against viraemia development if neutralization titres of 1:8 or higher were achieved[537]. Vaccinating sows with a booster dose of an inactivated vaccine before farrowing resulted in more piglets with neutralizing titres >1:32 at weaning and a delayed incidence of infection during the firsts weeks of life, suggesting a protective effect[595]. Similarly, Hsueh et al.[596] found a significant correlation between the maternally-derived neutralizing antibody titres, delayed infection onset, viral loads, and piglet survival.

In terms of potential interference with the development of active immunity after vaccination, several studies have indicated that interference may occur when MDA contain neutralizing antibodies for the vaccine[597,598]. However, this interference may not happen when these antibodies are not present[599].

1.7. PRRS EPIDEMIOLOGY

After the emergence of PRRSV during the 1980 and 1990 decades, the infection spread to most pig-producing countries worldwide. By the end of the 1990s, PRRSV-2 was present in all the countries of North America, as well as in Colombia, Chile, and probably other countries in South and Central America, along with Japan, the Republic of Korea, and China. Concurrently, PRRSV-1 spread to most European countries, except for Sweden, Norway, Finland, Ireland, and Switzerland. Denmark faced dual infections of PRRSV-1 and PRRSV-2, originating from an incident involving the spread of a vaccine virus. Although precise data were not available at that time, most Central and Eastern

European countries were likely infected as well. Australia and New Zealand have always been PRRSV-free. The virus was introduced occasionally and promptly eliminated in Sweden and Switzerland[600,601]. Currently, in most Asian countries, both PRRSV-2 and PRRSV-1 coexist. Only two countries, Chile in 2013 and Hungary in 2022, successfully eradicated the infection once established[16,17]; however, PRRSV was reintroduced to Chilean herds two years later.

Transmission of PRRSV can occur by both vertical and horizontal pathways. Vertical transmission happens in late gestation and plays a crucial role in the maintenance of the infection within the herd. Horizontal transmission can occur through direct or indirect contact between animals. In the upcoming sections, we will review both vertical and horizontal transmission, along with the excretion and transmission routes of PRRSV, exploring how it spreads within farms, as well as its transmission between farms (further reviewed[602,603]).

Noteworthy, the transmission of a virus is a fundamental event determining its persistence in the herd, shaping its genetic diversity, and exposing it to selective pressures and to adaptation to new hosts, as it is further reviewed in *Section 1.10. PRRSV evolution and genetic diversity*.

1.7.1. Vertical transmission of PRRSV.

Vertical transmission from sows to their offspring is a key element in the epidemiology of PRRSV. Published evidence indicates that vertical transmission occurs mainly, if not exclusively, in late gestation. The most widely accepted explanation is that before day 80 of gestation the number of highly susceptible CD163/CD169 double positive macrophages is scarce. However, it is not known how the virus crosses from the maternal to the foetal side of the placenta (further reviewed[360]).

In any case, vertical transmission can result in the delivery of infected piglets that can remain viraemic for a prolonged time. In fact, after experimental inoculations during late gestation, between 20 and 100% of viraemic piglets have been reported at birth[400,402,406,423,424,604–606]. Moreover, born-infected animals may persist infected for long periods and may contribute to the viral circulation within the herd for extended periods of

time[369,370,373,375,566,607,608]. Infectious PRRSV particles have been identified in lymphoid organs of born-infected pigs up to 132 days of age and they have been shown to shed and transmit the virus to naïve pigs for up to 112 days of age[423,424]. Therefore, these animals are considered the primary carriers of infection, contributing to the spread of the virus to their siblings or pen-mates within both farrowing units and nurseries and, in turn, contributing significantly to the perpetuation of the infection within the herd[606].

In the field, the proportion of viraemic litters in an unstable farm can be variable. According to Vilalta et al.[609], farrowings with birth of viraemic piglets can be identified for as long as twenty-three weeks following the initial outbreak. Young sows seem to have a higher likelihood to produce vertical transmissions even when vaccination is performed[609,610].

1.7.2. Horizontal transmission of PRRSV.

Pigs may be infected by PRRSV through direct contact or indirect exposure. The virus enters the host through oronasal, intramuscular, vaginal, and intrauterine routes, and transmission may occur at least through insemination/coitus, inoculation, inhalation, and ingestion[404,611–617].

The minimum infectious dose (MID) for the horizontal transmission differs depending on the exposure route, with different studies reporting varying infectious doses 50 (ID_{50}) values for oral, nasal, intramuscular, aerosol, and sexual exposure[617–619]. Percutaneous exposure, often occurring during farm practices like ear notching and injection, has the lowest MID (around 10^1-10^2 50% tissue culture infectious dose ($TCID_{50}$)), and may occur through contaminated needles, bites, cuts, and abrasions during aggressive pig behaviour[375,617,620]. Transmission by aerosol requires about 10^4-10^5 $TCID_{50}$[621,622]. ID_{50} values also seem to depend on the viral strain. In general, the higher the virulence, the lower the MID[621,622]. Recently, ingestion of contaminated feed material has been also identified as a source of infection[623].

Direct contact represents the most effective pathway for the transmission of PRRSV and may occur through oral and nasal secretions[373,404,436,620], semen[438,611,624,625], mammary gland secretions[626,627], urine[436,620], and faeces[362,404,436]. The duration and level of

viral shedding depends on the route of shedding and the viral strain[366,404,620,628,629]. Transmission by direct contact from infected gilts to sentinels has been documented up to around eighty days post-inoculation[375].

In oral fluids, positive results for viral shedding have been described early in the infection, between one to four days post-infection[629,630]. Studies have shown relatively constant shedding in oral fluids, with the virus being isolated from pigs for an extended period of time, up to forty-two days post-inoculation[620,631]. In both cases, the virus continues to be excreted through this route beyond viraemia, albeit intermittently[620,629,632].

Nasal excretion also starts early in the infection, peaking between two and seven days, and decreases steadily thereafter, until up to forty-eight days post-infection in piglets, yet it has also been described to vary among individuals and viral strains[365,633,634]. In general, PRRSV strains of moderate or low virulence seems to have a limited replication in the nasal mucosa and nasal shedding is intermittent and with low viral loads[404,436,612,635]. In contrast, highly virulent strains seem to result in long nasal shedding and high viral loads in the nasal secretions[103,633,635].

In semen, PRRSV has been detected up to ninety-two days post-infection, with intermittent shedding described[438,624,625,630,636,637]. Very few studies have examined the replication of PRRSV in the male reproductive tract. Sur et al.[521] showed that the virus presence in the testis was associated to viraemia, suggesting that presence of infected macrophages is the main driver of seminal shedding. However, the presence of infected macrophages can be prolonged. The virus has been isolated in different boar reproductive tissues up to 101 days post-infection[362,625,638]. Viral shedding in semen occur in non-viraemic and seropositive boars[624,625,637,639]. The shedding pattern, whether constant or intermittent, has also been suggested to depend on the strain and virus dose of exposure[640].

Indirect contact also plays a significant role in PRRSV transmission. Contamination of footwear and coveralls[641] or transport vehicles[642] with body fluids from infected animals can also act as a source of infection. The role of the fomites in PRRSV transmission is more substantial during colder weather conditions, as opposed to warmer conditions, because of the higher viability of the virus in winter conditions[643,644]. Contaminated needles have also been shown to transmit PRRSV[645]. The potential for the virus to persist on fomites highlights the importance of addressing indirect transmission routes in

biosecurity measures. It emphasizes the need for thorough cleaning and disinfection protocols, not only for animal-related elements, but also for surfaces and objects involved in farm operations, to minimize the risk of PRRSV spread.

PRRSV might potentially be transmitted by flies or mosquitoes, acting as mechanical vectors[646,647]. For example, houseflies have been shown to carry PRRSV in the surface and intestine tract and transport the virus up to 2.4 km away in controlled field conditions[648–653]. However, it is thought that mechanical vectors play only a secondary role in within-farm transmission and, if any, a very limited one in between-farm transmission.

1.7.3. Infection cycle and within-herd transmission.

When PRRSV enters a naive breeding herd, initially causes a reproductive outbreak that rapidly spreads among sows. During this phase, vertical transmission is predominant. Most piglets in the farrowing units become infected, either by *in-utero* infection or through horizontal transmission by direct contact or through milk. These infected piglets will further spread the infection in nurseries, and, at this stage, respiratory disease will develop. The impact of the respiratory outbreak depends on several factors, including the virulence of the PRRSV strain, the presence of other respiratory pathogens, and the conditions in which animals are raised.

The epidemic stage in sows unfolds until the density of susceptible animals decreases, which takes approximately between one to four months from the onset of the outbreak if no control measures are implemented[425,433]. Then, the farm enters in an endemic phase in which the disease is more insidious, with suboptimal reproductive performance and recurrent respiratory disease in nurseries. The virus can be maintained through transmission between sows, a flow of viraemic piglets from the farrowing units to the nurseries, and by retrograde transmission from nurseries to farrowing units [654].

In very small herds, where the entire population may achieve near-simultaneous immunization, the virus may potentially fade out[655,656]. However, in larger populations, subpopulations of animals that remain uninfected contribute to maintain the infectious cycle and to a sustained low-level of viral circulation[656,657]. For instance, PRRSV could be detected in nursery pigs even 3.5 years after the initial outbreak[425,658]. Over time, the

combination of introducing susceptible animals (such as inadequately acclimatized gilts) and the decline in the specific immunity, in the absence of re-exposure or vaccination, leads to an increase in the proportion of susceptible animals. Once the density of susceptible animals reaches a threshold (which is unknown), it sets the stage for a new outbreak[656]. This cyclical pattern typically unfolds over 1.5 to 3 years[655,656].

Young-infected pigs may further contribute to the virus's circulation within the herd, since they harbour the virus at low levels and for extended periods of time[369,370,373,375,566,607,608]. Infectious PRRSV particles have been identified in lymphoid organs up to 157 days post-infection in four-week-old piglets[373]. Hence, the critical point in PRRSV control within a herd, and the foremost priority, is to interrupt the viral circulation among sows, preventing vertical transmission and the birth of infected piglets. This can be attained with a combination of measures that at least include good biosecurity practices, vaccination, and a correct management of the pig flow.

Both the size of the herd and the location and contact between animals within the farm influence the persistence of PRRSV within a herd[654,655]. Nodeljik et al.[655] reported an estimated infectious period of fifty-six days following exposure and calculated that six years were required to eliminate PRRSV from a 115-sow herd without lateral introductions and eighty years for a 230-sow farm.

1.7.4. PRRSV transmission between herds.

Multiple routes of transmission contribute to PRRSV spread between herds, being the movement and introduction of infected animals one of the main ones[659–665]. The highest risk scenarios involve introducing infected replacement gilts into reproductive herds or comingling susceptible and infected weaned piglets from various sources into fattening units. Replacement gilts have been reported to serve as a significant source of virus introduction, especially when introduced from an external herd with an unknown or positive PRRSV status[661,666,667]. As mentioned before, infected gilts can transmit PRRSV for up to three months. Pesente et al.[668], based on phylogenetic analysis, indicated that the source of infection in farms with a persistent PRRSV status was most likely the introduction of infected animals, rather than from the emergence of escape mutants in the circulating strains.

Contaminated semen is considered one of the main sources of PRRSV spread[659–661]. This source has a great potential for disseminating PRRSV, as semen from an infected boar nucleus can be used for artificial insemination in hundreds of farms. This has been evidenced in outbreaks in Denmark and Switzerland[601,669].

In the present context, the broad interconnectivity among modern pig farming companies, particularly within large multisite companies, has become a significant factor facilitating the spread of PRRSV. Transport vehicles have emerged as a key element of transmission. Galvis et al.[665] showed that trucks particularly those that transport feed, has a high potential to spread PRRSV. Additionally, sharing service providers and other interconnected activities between farms contribute to the dissemination of the virus[600,662,665,666,670,671].

A special case is long distance airborne transmission. It has been shown that pigs may become infected by inhaling aerosols for both short and long distances, being 9.1 km the farthest between-farm transmission distance documented[672–678]. However, data on airborne transmission are somehow conflicting and some studies indicate that it is not a frequent mode of transmission and represents a low risk[661,665–667,670,679,680]. Probably, the discrepancies arise from the use of different strains and experimental conditions. It was shown that the likelihood of PRRSV airborne transmission is influenced by factors such as the weather, the environmental conditions[679,681] and the viral strain[628,672,675]. Strains with increased replication in the nasal mucosa would have higher chances to be spread by airborne transmission[633].

The risk of PRRSV transmission between herds is also influenced by factors such as herd density, the proximity of a herd to other infected ones, herd size, and pig density[658,661,662,664,682–684].

1.8. PRRS DIAGNOSIS

Diagnosing PRRSV involves a combination of clinical observation and laboratory testing, with techniques such as reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) commonly employed for accurate detection.

1.8.1. Diagnostic methods for detecting PRRSV infection.

Diagnosing PRRSV based on clinical signs proves unreliable due to the wide variation in the manifestations among herds and individual pigs, particularly in endemic phases and because of the lack of any pathognomonic sign or lesion.

Hence, the diagnostic approach should consider the clinical signs, the clinical history, and alterations in production records; however, confirmation of PRRS diagnosis requires diagnostic tests (further reviewed[433,685,686]). Briefly, as outlined in the upcoming text, currently, RT-PCR is the most employed method for detecting the virus, while ELISA is the standard for identifying antibodies against PRRSV. The combined use of RT-PCR and ELISA proves effective in detecting PRRSV infection, determining its occurrence, and monitoring viral circulation and the immune status of the herd. Additionally, sequencing is increasingly being employed, offering valuable insights such as identifying the potential source of the virus. While ORF5 Sanger sequencing has traditionally been the primary method, there is a growing trend towards utilizing next-generation sequencing (NGS) for whole-genome sequencing (WGS).

1.8.1.1. RT-PCR and other techniques aimed at the detection of the virus.

Both endpoint RT-PCR and the quantitative real-time RT-PCR (RT-qPCR) are employed for PRRSV detection. These methods rely on the detection, amplification, and, in RT-qPCR, quantification, of a specific segment of PRRSV genome, such as nsp3, nsp9, ORF5, and ORF7[687–690]. The most commonly used RT-qPCR is based on ORF7 detection, as it is a well-conserved segment of the genome, is the most expressed segment in the viral replication, and allows differentiation between PRRSV-1 and -2[691–694]. Noteworthy, while positive RT-PCR does not equal to the presence of viable virus in the sample, there exists a strong correlation between RT-PCR results and the identification of replicating virus[636].

Besides, it is worth noting that the virus, and its RNA, is rapidly degraded in autolytic tissue[77,368]. As a result, RT-PCR may yield negative results when used with samples obtained from mummified foetuses, even if the abortion is caused by PRRSV[414]. False negative results can be also obtained from semen samples because of the presence of PCR inhibitors in semen[636]. A third source of false negative results arise from the high

genetic diversity of PRRSV that may produce mismatches between the primers and the actual sequence in the viral genome. This underscores the need for regularly updating the primers used in RT-PCR to minimize the risk of obtaining false negative results[35,695–697].

Other methods to detect the virus include virus isolation[19,87,698,699], loop-mediated isothermal amplification (LAMP)[700–703], immunohistochemistry (IHC)[704,705] and in-situ hybridization (ISH)[706,707].

Viral isolation can be performed using PAM or continuous cell lines such as MARC-145 or CL-2621, both a subclone of the line MA-104 that has been identified as green monkey (*Chlorocebus pygerythrus*) kidney[19,75,87,698]. Some immortalized macrophage cell lines have also demonstrated good results[90,122]. If the samples are adequate and have been preserved in refrigeration or frozen, isolation is most often successful in PAM[698]. In contrast, isolation in the aforementioned cell lines is only successful in a low proportion of cases. In a comparative study using a pig macrophage cell line and MARC-145, the isolation rates in macrophages were significantly higher regardless of the type of sample[90]. Interestingly, the susceptibility of the green monkey cell lines to PRRSV infection has been related to a number of mutations in the envelope glycoproteins of the virus, suggesting that the success or failure in the isolation is a matter of how well the mutated strain interacts with the alternative receptors of the cell lines[708,709]. Moreover, viral adaptation to MARC-145 cells has been reported to lead to mutations in both non-structural and structural proteins[710–712].

LAMP is a convenient alternative to RT-PCR when the limitation of resources is critical. Being an isothermal amplification, it does not require any sophisticated equipment. LAMP may be transformed in a visual colorimetric test. Several reports suggest that LAMP is a promising technique for on-field application[702,703].

Immunohistochemical detection of the virus in tissues is a good option for the diagnosis of PRRSV in cases of respiratory disease, as it allows the co-localization of the virus with the lesions. However, IHC seems to have lower sensitivity unless several sections of tissue are examined[713]. In addition, the duration of the fixation is a critical factor for the sensitivity. In previous studies it was shown that fixation times longer than twenty-four hours resulted in significant losses of sensitivity[714]. In contrast, in situ

hybridisation seems to be more sensitive than IHC but also requires a good preservation of the RNA integrity[715].

1.8.1.2. Serological tests.

As for the assessment of antibodies, ELISA is widely used for herd-level infection detection and monitoring[716,717]. Currently, there are many commercially available ELISA kits for the detection of anti-PRRSV antibodies, mainly against the N protein. Animals usually seroconvert rapidly and anti-N antibodies remain detectable for several months[526,527,531,532].

The ELISA tests are valuable for detecting seroconversion, assessing PRRS status of replacement gilts, conducting seroprofiles by analysing sample series from various age groups to monitor viral circulation and the age of infection, determine duration of MDA, or assess vaccine efficacy. However, noteworthy limitations include the inability of current ELISA tests to distinguish between infected and vaccinated individuals, as well as the lack of correlation between detected antibodies and protection. Moreover, negative or weakly positive ELISA results do not necessarily exclude the possibility of persistent infection or lack of immunity[718,719]. Furthermore, important sensibility and specificity differences of the available kits have been reported[720–724].

In the context of antibody detection, other approaches encompass viral neutralisation test (VNT)[535,548], immunoperoxidase monolayer assay (IPMA)[18,725,726], immunofluorescence assay (IFA)[526,727], and fluorescent microsphere immunoassay (FMIA)[723,728]. Of these, the VNT is of importance for the assessment of protection as several reports indicate that VNT titres of ≥1:8 may result in protection against the infection[537,556] The available evidence indicates that neutralizing antibodies often have a very limited capability to neutralize heterologous strains[551]. VNT requires the adaptation of the viral strain to MARC-145 or an equivalent cell line. This is a problem because some isolates are very difficult to adapt or cannot be adapted at all to this cell line. The use of PAM for the VNT presents problems of standardization and reproducibility of the results. Recently, it has been reported the use of a macrophage immortalized cell line for this purpose with apparently good results[729].

1.8.2. Diagnostic approaches.

In the preceding sections, we revised the different techniques that can be used for the diagnosis of PRRSV infection. However, the mere availability of diagnostic techniques is not sufficient. A systematic and rational diagnostic approach is crucial for successful diagnosis and monitoring of PRRSV infections, which will be further reviewed in the following sections.

1.8.2.1. Reproductive disease.

The diagnosis of PRRSV abortion in sows can be conducted through the examination of the sow or the aborted foetuses. Abortion typically occurs within a period ranging from a few days to three to four weeks following the initial infection, although it most commonly happens around days 5 to 15[8,403,417]. Considering the duration of viraemia in sows, which is typically up to two weeks, a significant proportion of sows will be viraemic at the time of abortion. Collecting blood samples from the aborted sows and subjecting them to RT-PCR analysis is likely to yield virus detection. However, due to the varied timeframe within which abortion can occur after the onset of infection, it is conceivable that a specific animal may no longer be viraemic. Therefore, examining multiple sows enhances the likelihood of detecting the infection.

A second approach is testing the aborted foetuses. The primary advantage of this method is that a positive result can be considered conclusive evidence of the abortion's aetiology. While RT-PCR remains also the most sensitive technique for aborted foetuses[730], the degree of autolysis in the foetuses can pose challenges during sample processing, and the potential for RNA degradation must be taken into account. Also, it must be kept in mind that not all aborted foetuses are infected. Once again, the more animals examined, the higher the chances of detecting the virus.

The third approach involves testing newborns before colostrum intake or stillbirths. Serum samples from these animals are highly valuable for demonstrating vertical transmission of PRRSV. Umbilical cords are also suitable samples for this testing[731]. However, caution is advised when interpreting the results regarding the confirmation of the aetiology of a reproductive outbreak in an already PRRSV-infected herd. In many herds,

PRRSV may lead to vertical transmission events without overt reproductive signs. In most instances, these will be complementary data to those obtained in the examination of sows and foetuses.

1.8.2.2. Respiratory disease.

Given the multifactorial nature of the porcine respiratory disease complex, it is crucial in a specific case to ascertain whether PRRSV is the primary triggering agent, upon which other agents may complicate the course, or if there are additional primary agents involved. To achieve this, combining the detection of the virus with the histopathological examination of the affected lungs is essential. This can be accomplished using RT-PCR or IHC for virus detection (note that the limitations of the latter technique have been detailed before). Collecting blood samples of pigs may prove useful in identifying viraemic animals and determining critical points of viral circulation. Specifically, it helps ascertain whether PRRSV is circulating in farrowing rooms or if circulation is initiating in the nurseries.

1.8.2.3. Boar and gilt monitoring.

Monitoring boars presents a complex challenge. Infected boars have been described to shed PRRSV in semen for a duration ranging from three to ninety-two days, and this shedding may be intermittent, with or without viraemia[624,625,637,639]. This intermittent shedding is suggested to be due to PRRSV replication in the macrophages within the genital tract, since replication in germinal cells in the testes has been only demonstrated during the viraemic phase[521,639]. An additional problem arises from the presence of PCR inhibitors in semen, which can lead to false-negative results.

Considering the dynamics of the infection in boars and the limitations of semen testing, the most effective approach to boar monitoring involves a combination of serology and RT-PCR[637]. RT-PCR is adept at detecting viraemic animals or those actively shedding the virus in semen. Simultaneously, serology confirms the absence of anti-PRRSV antibodies in the herd, enhancing confidence in the analysis.

Monitoring gilts is essential for effective PRRSV control[732]. In addition, inadequate acclimatization of PRRSV-naïve gilts may further create a subpopulation of non-immune

animals that can fuel the transmission of the resident PRRSV strain. Consequently, thorough monitoring of PRRSV both upon the entry of the gilts in the farm and before introducing them into the breeding herd to confirm no viral shedding and validate the success of acclimatization, becomes paramount[733].

Upon introducing gilts during the quarantine period, it is advisable to assess their PRRSV status through an approach combining both PCR and serology for maximum effectiveness. If gilts come from a PRRSV-negative source farm, they should be serologically and PCR negative. On the contrary, if gilts come from a positive source whether due to vaccination or not, only PCR testing is mandatory. However, it is important to note that animals testing PCR-negative in serum may still be infected and potentially infectious, posing a risk for introducing the virus. Therefore, introducing seropositive animals into a PRRSV-negative herd is extremely risky, but introducing seropositive external replacements into already PRRSV-positive herds also carries risks.

One of the main purposes of gilts acclimatization process is to facilitate the development of immunity against pathogens present in the herd, particularly PRRSV. This can be achieved through vaccination or exposure of sows to the virus present on the farm. However, the latter carries a high risk as it lacks control over the efficacy and timing of the exposure. This increases the probability of introducing gilts that are still contagious with the wild-type virus into the breeding herd. In any case, before introducing them into the breeding herd, it is essential to verify that the gilts have successfully developed an immune response, usually assessed by ELISA, and confirm that they do not shed virus, to prevent both the introduction of seronegative and viraemic gilts, respectively[733].

1.8.3. Pooling, aggregate samples, and alternative sample types for monitoring PRRS.

Classically, the established standard method for surveillance and assessing the PRRSV status of a herd involves collecting serum samples from a selected group of individuals (thirty to sixty), most often piglets at weaning[734,735]. For most farms, this approach is effective to determine the herd status when the incidence of the infection in the farrowing units is relatively high, regardless of the number of sows. However, in big farms, that often are operated with periodic farrowing batches, selecting thirty to sixty piglets for

testing does not necessarily reflects the complexity of the farm. When viral circulation in the farrowing units is limited (less than 5-10% of piglets testing positive), the mentioned sampling scheme is insufficient to detect the viral circulation. Therefore, sampling methods allowing a higher sensitivity are desirable. In such cases, alternative population-based methods, such as pooling strategies or analysis of aggregated samples have gained popularity. In addition, testing samples such as oral fluids or post-mortem samples can reduce piglet manipulation, aligning with animal welfare concerns. Costs reduction and simplification of the sample collection process are additional benefits. However, individual samples remain necessary for PRRSV isolation and sequencing due to technical challenges associated with sequencing from pooled or aggregated samples.

1.8.3.1. Sample pooling.

Sample pooling is a strategy consisting of mixing samples from different individuals and nowadays is commonly used for PRRSV monitoring. This method allows to reduce the number of tests and analyse a higher number of animals, reducing analysis costs.

Nevertheless, it is important to consider that mixing of positive and negative samples may result in a lower sensitivity compared to analysing individual samples if the pooling involves more than five samples. Considering the analytical sensitivity of the PCR and the average viral loads found in sera, pooling of less than five serum samples does not significantly affect the diagnostic sensitivity[736]. For example, Gerber et al.[630] showed that when individual samples yielded cycle threshold (C_t) values below 35, pools of five samples resulted in positive results within the detection limit of the PCR (C_t <37). Lebret et al.[737] observed a greater decrease in sensitivity when pooling five serum or oral fluid samples, which they attributed to the collection, processing, and storage procedures when samples are collected under field conditions. However, those small differences did not affect the detection of the infection in a pig batch. In another study, Lebret et al. [738] reported that pooling of family oral fluids should be avoided because of the lower viral loads. Vilalta et al.[739] showed that pooling of processing fluids is possible when samples had cycle threshold (C_t) values under 30.

The reduction of the sensitivity caused by pooling can be offset by analysing a higher number of samples, especially in cases of low prevalence, because this results in a higher herd-level sensitivity. Retesting individual samples from pools with C_t values close to the cutoff is recommended.

1.8.3.2. Oral fluids and other samples.

Aggregated samples are those that are obtained from different animals but in a single sample. The classical example of this in PRRSV monitoring is collective oral fluids[740–742]. Oral fluid is a combination of saliva and oral secretions, and it can be collected either individually or collectively from piglets, sows, and boars[629,630,632,743,744]. This strategy takes advantage of the natural curious behaviour of the pigs, and it is easy and a welfare-friendly process.

In general, collection of oral fluids is performed by hanging a rope in the pen and letting the animal time enough to explore it and chew it. The oral fluid-soaked rope is squeezed to extract the collected oral fluid, which is then centrifuged and analysed, usually by RT-qPCR. Cotton ropes are recommended for collecting oral fluids, although it is essential to consider that the material used may influence the results when antibodies are to be tested[629,745]. Prior exposure to the rope for sixty minutes is recommended for optimal sample collection success[629,743,746]. For pigs previously exposed, an adequate sampling period for participation is 20-30 minutes[743,747]. Extending exposure beyond thirty minutes offers no additional benefits[746].

Although oral fluids can be collected from individuals, the most common strategy is the collection of collective oral fluids in either nursery pens or in farrowing crates, the latter often referred to as family oral fluids. The size of the group can also affect pig interactions with the rope, and a single rope is recommended for seventeen to twenty-four animals[747,748]. Approximately 76.5%-80% of pigs interact with the rope[747,748]. For collecting oral fluids from individual animals, training is necessary[632,749].

Oral fluids should be promptly refrigerated or frozen to prevent RNA degradation[750,751]. There is controversy regarding the centrifugation of oral fluids. While Gibert et al.[751] reported that centrifugation at 15,000 x g for fifteen minutes increased the sensitivity, Olsen et al.[745] do not recommend centrifugation.

Individual oral fluids have a lower detection rate than serum samples[630,737]. PRRSV can be detected in oral fluids from one to four days up to forty-two days post-infection, and it may continue testing positive beyond the viraemic period[629–632]. During viraemic phases the use of oral fluids for RT-qPCR analyses results in similar or lower sensitivity than the use of blood samples; however, it is capable to detect positive animals after the cessation of viraemia[629].

Pen-based oral fluids allow sampling a larger number of pigs, increasing the likelihood of detecting PRRSV, particularly when it is in low prevalence[737]. Prickett et al.[752] reported a 77% agreement of PRRSV detection between oral fluids and sera at the pen level. Biernacka et al.[753] showed that oral fluids had higher pen-level sensitivity than pooled samples of four sera for detecting PRRSV. Kittawornrat et al.[754] observed that oral fluids were efficient to detect the presence of infected pigs in farrowing units with low prevalence (1.5%).

As explained above, oral fluids can also be used to detect the presence of anti-PRRSV antibodies. The antibody kinetics in oral fluids resemble those in serum. IgM and IgA appear at six to seven and seven to eight days post-inoculation, peaking at ten to twelve and ten to fourteen days, respectively, and then decreasing rapidly. IgG appear at eight to nine days post-inoculation, reaching a plateau from at fourteen to nineteen days[755,756]. Several studies have demonstrated a high sensitivity and agreement between ELISA IgG antibody levels in serum samples and oral fluids from individual samples, sample pools, and pen-based samples[632,744,753,757]. However, lower specificity has been described in some studies[723,744,758].

Family oral fluids have also been proposed for monitoring PRRSV in farrowing units. In this case, the rope must be accessible to both the sow and the piglets. Piglets imitate the sow chewing the rope, increasing thus the chances for a successful oral fluids collection[738,746,759]. Likewise in nurseries, allowing the animals a thirty-minute interaction with the rope after previous training yields a high success rate (89%)[746]. The addition of flavour to the rope can enhance success rates[746].

Almeida et al.[759] observed a 96.5% agreement between serum samples and family oral fluids for detecting PRRSV at the litter level. To enhance PRRSV detection, it has been suggested to sample high-risk litters based on parity (primiparous sows) and litter size (sows ≤11 piglets).

1.8.3.3. Processing fluids.

Processing fluids represent another type of aggregated sample that proves valuable in monitoring vertical transmission or viral circulation in newborns. This sampling method consists of collecting docked tails and testicles from castrated piglets and recovering the serosanguinous exudates produced. The popularity of this sample type has grown due to its practicality, ease of collection, and cost-effectiveness, allowing the analysis of a large number of litters[609,739,760–762]. However, in the European context (Directive 2008/120/CE), surgical castration and tail docking are practices that, although still allowed under certain conditions, are to be avoided, and it is not difficult to foresee that they will be banned in the near future.

The use of processing fluids is considered an effective method for herd classification and PRRSV monitoring, particularly in cases of low prevalence (<10%), and for farms working towards PRRSV elimination[609,760,761,763]. Combining these samples with blood samples has been recommended to enhance the sensitivity of the monitoring program[761,764]. Vilalta et al.[609] reported a sensitivity of 87% when at least one pig in the litter was positive, while Lopez et al.[762] reported a sensitivity of 73%. In another study, Vilalta et al.[765] compared the sensitivity among testicles, tails, and udder wipes, demonstrating that testicles exhibited higher sensitivity (92%) and higher agreement with serum samples (87%), compared to tails and udder wipes.

The possibility of pooling processing fluids to reduce costs has been explored[739,766]. It is recommended to analyse fluids from multiple litters concurrently instead of individual litter samples, as it increases the likelihood of detecting PRRSV[762]. In theory, a positive piglet can be detected in a sample from 50 negative litters (approximately 600 piglets)[739]. Lopez et al.[766] estimated that by grouping samples from up to 30 litters (approximately 320 piglets), a 95% confidence in detecting PRRSV is achieved, when the prevalence in piglets was close to zero. Furthermore, IgG in processing fluids can also be analysed[760,767].

1.8.3.4. Tongue tips.

Monitoring through tongue tips of dead animals has recently emerged as a potential alternative sampling method[768]. This method involves collecting the tongues of the dead animals present in the same room, placing them in a single bag. Then, samples are frozen

and thawed, and the exudating fluid is collected and analysed by RT-qPCR. This type of sampling has been proven valuable in scenarios with low prevalence of PRRSV in breeding herds and nurseries[768]. The use of tongue tip exudates seems to be more sensitive than sera to detect PRRSV at the farm level. Since PRRSV-infected animals are more likely to die, tongue tips recovered from dead animals are more likely to include infected animals. Machado et al.[769] noted that the correlation of results between tongue tips and sera was very high, close to 95% sensitivity and 100% specificity taking sera as the gold standard. However, in one-day old piglets C_t values are lower in tongue exudates than in sera[769]. Notably, high quality ORF5 sequences were obtained from tongue tips[768]. Collectively, these findings suggest that tongue tips could serve as a method for PRRSV monitoring, although further research is required for validation.

1.8.3.5. Udder skin wipe.

Udder skin wipes from lactating sows are also another approach to collect indirectly aggregate samples, since udder skin may be contaminated by oronasal secretions of the piglets. Vilalta et al.[770] showed that PRRSV could be detected in both udder and surface wipes within the first three months following an outbreak, and sensitivity reduced from 56.25 to 14.3% from three to six months after the outbreak. Udder skin wipes, anyway, have lower sensitivity compared to serum samples, processing fluids, and family oral fluids[738,765,770], and some authors consider that it is not adequate for PRRSV surveillance[738].

1.8.4. Sequencing.

Sequencing plays a crucial role as a complementary tool in PRRSV diagnosis, providing valuable insights into epidemiology. For instance, it proves useful for determining probable sources of infection, identifying circulating strains and their relationships, differentiating field-virus isolates from vaccines, determining the occurrence of lateral introductions, monitoring viral evolution, and detecting recombination events[277,378,379,573,686,771–781]. This information is instrumental for establishing effective control measures for PRRSV.

Phylogenetic analyses based on the obtained sequences contribute to predict relationships among isolates. Noteworthy, considering recombination is crucial when interpreting phylogenetic trees, as it can obscure phylogenetic relationships among strains[38,782]. Accordingly, an analysis of recombination must be performed together with the phylogenetic analysis.

Unfortunately, phylogeny does not allow making forecasts of the likelihood of cross-protection between strains or predict vaccine efficacy[449,569].

Until recently, the exploration of PRRSV genetic diversity relied on ORF5 Sanger sequencing[777,778], employing a widely accepted 97% similarity cutoff for strain differentiation[783]. ORF5 was selected due to its diversity and the presumed influence on both viral pathogenesis and immunity[39]. Sanger sequencing necessitates C_t values below 31-33, depending on the sample type[771]. Furthermore, its scope is limited as ORF5 only represents 4% of the total genome.

Besides, the construction of a whole-genome sequence using Sanger sequencing is very time consuming. Sanger sequencing rarely can produce reliable sequences of more than 600-700 nucleotides. For this reason, the construction of a whole genome of virus like PRRSV requires no less than twenty to twenty-five overlapping PCRs, which products need to be sequenced. In addition, Sanger sequencing yields consensus sequences that will necessarily neglect low frequency mutants in the quasi-species. Also, Sanger sequencing results become challenging in the presence of co-infections. Moreover, owing to PRRSV's high mutation rate, the set of primers required for segment amplification through PCR may not consistently match, demanding continuous revision and updating. Collectively, these factors render Sanger sequencing little practical for examining certain facets of viral evolution.

WGS using NGS addresses these drawbacks, providing a more comprehensive insight into PRRSV genetic diversity and viral evolution (further reviewed[784,785]). WGS not only improves precision in establishing phylogenetic relationships and its subsequent benefits but also allows for an effective identification of recombination events. Numerous NGS approaches have been employed for the examination of PRRSV[771–774,786–792].

Indeed, the application of these advanced methodologies, to date the so-called second-generation sequencing platforms (Roche 454, SOLiD, Ion Torrent, and Illumina),

has represented a tremendous leap forward in the study of PRRSV (further reviewed[793,794]). Additionally, with the decreasing cost of these techniques, they are becoming more used. However, they encounter challenges such as expensive equipment and *ex novo* genome assembly.

Illumina stands out as the most widely used among second-generation sequencing technologies. This methodology generates relatively short reads, posing challenges for the *ex novo* assembly. Besides, the inability to obtain long reads complicates the analysis of the quasi-species composition in viruses like PRRSV, as it is impossible to of generate a full genome sequence, or at least a sequence long enough, from a single viral particle. Illumina's approach provides base-by-base sequencing, with a high sequence accuracy. Other advantages are a high sample throughput and the lowest per-base cost among the other second-generation sequencers. However, Illumina has limitations, for instance, in detecting rare variants, particularly those occurring at a frequency below 1%. Another drawback is the extended sequencing times it entails compared to the next method.

Nanopore sequencing, a third-generation technology, is promising for field applications and real-time pathogen detection, particularly the MinION device, boasting portability and affordability. Notably, it sequences single molecules at real time and offers ultra-long raw reads. Nevertheless, the main concern of this method is the high sequencing error rate compared to other technologies[794–796]. This methodology has been recently used to sequence PRRSV[797–800].

The high throughput of the NGS methodologies enables a thorough characterization of the diversity within viral quasi-species, encompassing variants present at low frequencies. For PRRSV NGS, a C_t value below 25 is required[771], often requiring approaches to amplify the virus prior to NGS, which may introduce mutations in virus genomes. When assessing the outcomes, it is crucial to consider factors such as C_t value, overall quality of the samples, RNA integrity, and type of sample, as these can impact the analysis[771].

1.9. CONTROL OF PRRSV

Addressing the challenge of preventing the entry and circulation of PRRSV on a farm requires a multifaceted approach, as no measure is effective on its own. The pursuit of control and ultimate eradication relies on the interplay of four pillars: i) the implementation of stringent biosecurity measures to impede the entry and dissemination of the virus; ii) the employment of an effective surveillance plan to detect when and where the virus is circulating; iii) the adoption of management practices that curtail virus transmission within the farm and mitigate the impact of the disease; and iv) the establishment of a robust and consistent herd immunity to reduce the occurrence and severity of outbreaks (further reviewed[602,801,802]).

1.9.1. External biosecurity practices.

Biosecurity is usually defined as the group of measures and actions taken to avoid the entry and dissemination of new pathogens in the farm. Biosecurity encompasses at least two interconnected aspects: external and internal measures, each playing a crucial role in controlling PRRSV. External biosecurity focuses on preventing virus entry and involves measures concerning a) animals, mainly replacement gilts and semen; b) vehicles and visitors, including transport, cleaning protocols for trucks, control of visitors, and external materials; and c) managing the farm's proximity to other pig farms. Most external biosecurity measures entail establishing physical barriers to avoid, limit, or regulate the contact of animals, people, or vehicles coming from the outside. On the other hand, internal biosecurity focuses on limiting the spread of the virus if it has already entered the farm, involving animal management practices, personnel training, and diagnostic and record-keeping routines. Next, we will focus on the approaches to avoid the introduction of the virus into a herd.

1.9.1.1. Gilts quarantine and acclimatization.

Gilt introduction is one of the most critical factors to control, as they can introduce the infection, but can also be a source of susceptible individuals if not adequately acclimatized[803]. In fact, Pesente et al.[668] documented that the most frequent source of

PRRSV infection was through the replacement gilts. Therefore, it is imperative that when introducing external replacement gilts, the source is consistently negative[732]. This practice decreases the chances of introducing infected animals and allows the implementation of a well-planned immunization program.

Replacement gilts should undergo first a quarantine or isolation period and then they must be immunized before entering the breeding herd[732,804]. The purpose of the quarantine period is to avoid the introduction of infected gilts. Therefore, the duration should be sufficient for detecting any potentially infected animals or, in the case of purchasing gilts from PRRSV-positive sources, allowing the clearance of the infection. The length of quarantine is contingent on these factors. In the case of introducing animals from PRRSV-positive sources, whether vaccinated or not, and considering reports of transmission from infected gilts up to three months post-challenge, a recommended quarantine period would be ninety days[375,607,732]. When introducing animals from certified negative source, shorter quarantine periods may be acceptable. Regardless, diagnostic monitoring through both PCR and serology would be mandatory. Ideally, quarantine facilities should be located off-site or, if on-site, away from the breeding facilities[805]. These facilities should operate as a fully independent unit of the farm, with exclusive personnel, clothes, boots, and materials dedicated to the quarantine.

The acclimatization of gilts can be accomplished through various strategies, including vaccination or exposure to the farm's resident strain. Vaccination involves administering one or two doses of modified-live vaccine (MLV) to gilts before introducing them to the breeding herd. It is advisable to allow time for immunity to fully develop before introducing the gilts in the breeding stock. Usually, a minimum of four weeks after the initial vaccination is recommended.

Alternatively, gilts can be deliberately exposed to the circulating strain, by introducing them to viraemic animals, or by deliberated infection (usually injecting serum from infected animals)[806,807]. Although these methods have been used as an emergency action, they are not advisable because of the associated risks. When exposure is by contact, some gilts might not become infected, or infections may take place in different moments. As a consequence, the circulation of the virus within the gilt batch can be prolonged, with the potential risk of introducing infected gilts in the sows' stock. The deliberate infection by serum injection faces biosafety, ethical, and legal problems.

1.9.1.2. Semen.

Because PRRSV can be present in boar semen[625], boar studs and artificial insemination can potentially serve as a source of infection. Although the implementation of monitoring protocols and high biosecurity measures in these facilities, instances have occurred, as documented by Nathues et al.[601], who reported an outbreak in Switzerland following the use of imported semen. The critical preventive measure is to exclusively purchase semen from certified PRRSV-negative boars that are subjected to a rigorous monitoring program. Detecting the virus in semen by RT-PCR is considered unreliable because of its low sensitivity, being serum or oral fluids better samples[736]. In any case, intensive monitoring protocols are needed to detect PRRSV infection in a boar nucleus. Rovira et al.[736] calculated the probability of detecting PRRSV infection in a boar nucleus under different circumstances. In a 500-boar herd, even with testing sixty animals three times a week using RT-PCR, it would take no less than thirteen days to identify viral circulation if random sampling is employed.

1.9.1.3. Vehicles, personnel, and fomites.

Avoiding the entrance of people or vehicles into the farm premises is one of the key measures to prevent the introduction of PRRSV. Fundamental measures such as having a perimeter fence, a controlled access system, a load/unload deck, and a delimitation of clean and dirty areas are very helpful to prevent the entrance of PRRSV and other diseases. It is also important to have strict protocols for employees and visitors, such as a mandatory shower, plus require visitors to keep a downtime period before entering a farm[808]. Clothing and boots must be of exclusive use in the farm. The use of gloves and frequent hand washing is also recommended[641,809]. As far as possible, workers should be assigned to specific areas of the farm and prohibited from entering other areas, or at the very least, not without changing clothes, boots, and washing hands. If tools or other materials need to be introduced in the farm, it is recommended to ship all incoming materials with bag-in-box containers and ensure disinfection and decontamination upon arrival[809].

Appropriate cleaning, disinfection, and drying procedures are crucial for sanitizing transport vehicle [642,810–812].

Recently, it has been reported that contaminated feed may serve as a source of PRRSV infection[623]. The use of feed mitigants could prevent the possible introduction of the virus through this means[813].

To avoid transmission through house flies and mosquitoes, windows should be protected with screens to prevent insect entry, together with the use of insect bait and insecticides[814] .

1.9.1.4. Air filtration systems.

To minimize the risks associated with airborne transmission of PRRSV from neighbouring farms, a growing implementation of air filtration systems in swine facilities has been reported. Several studies have been performed to evaluate the efficacy of air filtration and it has been proven to be effective to prevent transmission between rooms, buildings, and farms in high pig density regions, reducing the number of PRRSV outbreaks[677,679,680,815,816]. In fact, farms lacking air filtration systems exhibited from three to twenty-fold increase in the likelihood of PRRSV introduction[680,813,817].

1.9.2. Assessment of herd status and monitoring of PRRSV.

For the successful control and eradication of PRRSV within the herd and implementation of national and/or regional programs, the assessment of their PRRSV status is crucial. The American Association of Swine Veterinarians (ASVV) approved in 2010 a herd classification system for describing PRRSV status, which has been widely used and recently modified[734,735]. The classification is based on the monitoring of the viral shedding and the exposure to the virus. Briefly, breeding herds are classified in the following four categories:

- Category I-A: Positive unstable, high prevalence. This category includes positive herds with a high prevalence of PRRSV-positive pigs at weaning, as well as those herds with unknown PRRSV status.
- Category I-B: Positive unstable, low prevalence. This status is attained when positive herds, after ninety days of testing, demonstrate reduced viral shedding and transmission in the farrowing units, with intermittent detection of PRRSV in

piglets' serum from birth to weaning. If the farm employs MLV vaccination and a sample yields a positive result, it will be considered negative if the virus is a vaccine strain as confirmed by ORF5 sequencing, WGS, or PCR clamping assays.

- Category II: Positive stable. PRRSV stability of a farm is achieved when the viral circulation ceases in the breeding stock, leading to the interruption of vertical transmission and subsequent birth of PRRSV-negative piglets. The herds in this status are characterized by consistent non-viraemic piglets at birth. Herds can be further classified to belong to the Category II-vx, which includes those implementing MLV to replacement gilts, sows, and/or piglets.
- Category III: Provisional negative. This is a transitional category for herds that are applying methods to eliminate PRRSV from the herd.
- Category IV: Negative. PRRSV-negative herds are those with no circulation of the virus and have to demonstrate that sows are PRRSV antibody-negative, and piglets are PRRSV-negative.

Growing and finishing sites are also classified in categories, which are: i) positive, ii) seropositive, non-shedding, iii) vaccinated, and iv) negative, by testing monthly 6 oral fluid samples[735].

Moreover, periodical diagnostic testing must be performed for a herd to remain in a specific category and to detect changes in the circulation of the virus that may indicate the lateral introduction of a new strain, issues with gilt acclimatization, or breaches in the internal biosecurity, among other causes.

1.9.3. Control of PRRSV within the herd.

The first goal to control PRRSV within a herd is to produce PRRSV-negative weaned pigs from the farrowing units, commonly referred to as "stabilization"[801]. Achieving PRRSV stability can improve productivity in breeding herds, attributed to the rise in the number of born-alive piglets and the reduction in pre-weaning mortality[818].

An approach to control PRRSV in the sow herd is the use of vaccination to maintain a homogeneous level of immunity (see *Section 1.9.5. Vaccination against PRRSV*). To minimise the impact of PRRSV in the farrowing units and nurseries, a

practical management strategy is the MCREBEL™ method, which stands for Management Changes to Reduce Exposure to Bacteria and Eliminates Losses[805,819,820]. The primary objective of this approach is to reduce the spread of PRRSV and secondary bacteria among piglets and minimize the exposure of PRRSV-infected piglets to pathogenic bacteria, the primary cause behind mortality. However, it is also highly recommended to include this method in eradication strategies to avoid recurrent recirculation of PRRSV in the piglet population.

The key management practices of MCREBEL involve avoiding or minimizing cross-fostering and movement of animals, reducing treatments, taking care of the smallest pigs, and promptly removing any sick individuals[805,819,820]. Strict adherence to an all-in/all-out management approach should be applied in both farrowing units and nurseries, avoiding animal movements between rooms[805]. It is also crucial to ensure proper sanitation, disinfection, and drying practices in these facilities before introducing new groups of animals. Adopting practices such as changing needles between litters or employing needle-free technologies can also contribute to controlling the virus's spread[645]. Lastly, maintaining a strict unidirectional flow of animals is highlighted as a fundamental aspect of this strategy[805].

1.9.4. Eradication of PRRSV.

The strategies employed in managing PRRSV depend on the desired outcome, whether it is eradication and maintenance of a negative status, or simply controlling the disease and achieving a PRRSV-stable status. Various approaches have been employed to eradicate PRRSV, such as total depopulation/repopulation, partial depopulation, test and removal, and herd closure.

i) Total herd depopulation and repopulation: This is a drastic approach that entails depopulating all animals from the farm, thoroughly cleaning and disinfecting the facilities, and introducing PRRSV-free animals[801]. While highly effective and fast, this method can be financially burdensome[821]. However, it has been applied to eradicate PRRSV in some countries, such as Sweden and Hungary[17,600].

ii) Partial depopulation: Partial depopulation, also known as nursery depopulation, is also aimed to interrupt PRRSV spread and consists of emptying the nurseries by moving nursery pigs to other facilities and clean up and disinfect the rooms[822,823]. Nursery depopulation prevents PRRSV transmission from older infected piglets to those recently weaned, resulting in mortality reduction and an improvement of post-weaning performance[822,824]. It proves highly effective for achieving eradication once the farm is stable. This method is commonly used together with herd closure, mass vaccination, and unidirectional flow of pigs[825].

iii) Test and removal: This technique consists of testing all the breeding herd and remove those PCR-positive or seropositive individuals from the farm. The test and removal method has proven effective and rapid in eliminating PRRSV in certain herds characterized by low prior seroprevalence, isolation from other farms, the use of PRRSV-negative replacement gilts, and an absence of clinical signs for at least a year (suggesting low or absent viral circulation). However, ensuring the complete elimination of all seropositive animals proves challenging, and the associated diagnostic and production costs are prohibitively high[803,826,827]. In the Hungarian eradication program, some farms were eradicated using this approach[17].

iv) Herd closure: This strategic approach entails suspending introductions of replacement gilts for a long period (several months) into an infected herd, thereby reducing the number of susceptible animals that could sustain viral circulation[801,828,829]. When this is coupled with exposure of all resident animals to PRRSV, allowing the development of herd immunity, by intentionally exposing animals to the homologous strain or vaccinating with a MLV, it is known as whole-herd exposure or load-close-expose[607,830,831]. The underlying premise of herd closure is that, despite PRRSV's capacity for causing a long-term infection, the immune system eventually eliminates the virus from all tissues. Even if persistently infected animals are still present, transmission will reduce significantly or even cease, since there a no susceptible animals remaining. Once all animals test seronegative, PRRSV-negative replacement gilts can be reintroduced.

Herd closure has proven to be effective and the most economical of all the eradication programs, making it the most used for eliminating PRRSV from breeding herds[821,829,830,832]. The time to stability, and subsequent duration of herd closure, has been reported to take from four to up to ten months, varying among herds, and is influenced by

factors such as the virus exposure method, prior immunity, virus strain, time since the last PRRSV outbreak, season of the year, and management practices[761,831,833]. Additionally, herd closure, when paired with vaccination and unidirectional pig flow is effective to stabilize farrow-to wean farms, even in high-density areas[834].

After successfully eliminating PRRSV from a herd, it is essential to uphold stringent biosecurity measures to prevent its reintroduction[831]. However, the success and duration during which the herd remains PRRSV-negative is hardly predictable. Holtkamp et al.[835] reported that among thirty-three PRRSV-free pig farms established through complete depopulation/repopulation or as new sites, 40% tested positive within one year, due to the introduction of a new strain. Kikuti et al.[836]**,** who followed 1,316 breeding herds from 2009 to 2021, documented that 17% of them achieved PRRSV-free status and remained as such for a median of two years. However, after eliminating PRRSV, new outbreaks happened in 23% of the breeding herds in one year after being declared PRRS-free[836].

Numerous documented cases have achieved successful PRRSV elimination by combining the mentioned methods, coupled with additional measures such as biosecurity protocols or vaccination. PRRSV has also been successfully eradicated on a regional scale. PRRSV was eliminated from the area of Horne Peninsula, Denmark, using a combination of whole-herd closure, vaccination with MLV, and internal biosecurity measures [832]. Hungary has achieved to eradicate PRRSV through the National PRRS Eradication Programme launched in 2014, which was based on the immunization of pigs, implementation of strict biosecurity measures, including importation of PRRSV-free pigs and mandatory quarantine protocols, and continuous serological and virological monitoring[17,837–839]. For positive small-scale or backyard pig farms and large-scale fattening farms, the PRRSV-elimination method was depopulation and repopulation and in some cases test and removal, while large-scale breeding herds could employ depopulation/repopulation, herd closure, test and removal, and/or vaccination[17,837–839]. Unlike herd closure, complete depopulation/repopulation and test and removal proved to be successful methods to eradicate PRRSV from these large-scale breeding herds[839].

1.9.5. Vaccination against PRRSV.

To effectively interrupt the spread of a virus within a farm, it is crucial to reduce the number of susceptible animals and establish preventive measures to hinder transmission during different stages of production. Hence, the strategic management of herd immunity becomes paramount.

In 1994, the first vaccines targeting PRRSV were introduced to the market[840]. Their role as indispensable tools for managing the infection became evident, albeit with certain effectiveness limitations. While these vaccines often help to mitigate the clinical and economic impact of the disease, vaccinated animals may remain susceptible to infection. As a result, vaccination has been primarily used to reduce PRRSV's impact, rather than prevent infection. Overcoming challenges such as the genetic and antigenic diversity of PRRSV, the unclear protection correlates, the partial heterologous protection, and the widespread prevalence of the disease in densely populated pig areas prove to be hurdles in achieving a greater efficacy (further reviewed[277,393,841–843]).

Currently, most commercially available PRRSV vaccines are MLV or attenuated vaccines, with less frequent use of killed or inactivated vaccines (IV). The following section will delve into both types of vaccines, their efficacy and safety, vaccination strategies, and novel approaches to develop vaccines against PRRSV, all of which have been extensively reviewed[449,840,844–848].

1.9.5.1. Modified-live vaccines.

MLV are the most commonly used vaccines. They are produced by adaptation and extensive continuous passaging of the virus in cell lines to decrease their capability for replication and virulence[840,848]. This method triggers mutations in the genome, initially some associated with cell adaptation and subsequently some with attenuation [710,849]. However, the mechanism behind PRRSV attenuation remains elusive, although some mutations in both structural and non-structural proteins have been proposed to be linked to attenuation[710,849–852].

Vaccination with MLV of previously unexposed pigs elicits a weak humoral and CMI response, but with a similar pattern than that of a natural infection[505,565]. The

production of non-Nab occurs between seven to twenty-one days post-vaccination, while the development of Nab is slow-paced, taking approximately four to five weeks, and their titres are low[505,534,561,565,567,853–855]. Some vaccinated animals have been shown to be clinically protected against challenge, even in the absence of NAb, as well as clearance of viraemia has been observed in animals, either without or prior to NAb detection[505,564,856]. Hence, the CMI response seems to play a significant role in the protective effects of MLV. Despite MLV-induced IFN-γ responses being weak, delayed (requiring at least three to four weeks), and fluctuating for several weeks, the appearance of IFN-γ secreting cells seems to coincide with the reduction of viraemia[505,565,853,855].

MLV vaccines provide protection against genetically homologous strains[857–859], yet only partial o no protection against heterologous strains[534,558,567,568,860,861]. Therefore, vaccines closely resembling field strains are considered more likely to be effective. Unfortunately, the degree of genetic similarity does not correspond to the level of cross-protection and is not a good predictor of vaccine efficacy[505,569,862–864].

Concerning the commercially available PRRSV-1 MLV, which all are derived from PRRSV-1 subtype 1, studies have indicated a greater partial protection against heterologous strains within the same subtype[505,582,860,865], including the highly virulent Italian strain PR40[866], compared to subtype 3 strains[564,863,867]. Moreover, their effectiveness in conferring protection against PRRSV-2 is observed to be more limited or non-existent[856,860,868–870]. In comparison, several studies have shown that PRRSV-2 MLV seem to provide a slightly better cross-protection against PRRSV-1 strains[863,871–873].

Nevertheless, the extensive genetic diversity of PRRSV potentially turns any virus exposure in the field into a heterologous challenge. PRRSV outbreaks have been reported in numerous cases despite herds being vaccinated[58,874–876]. However, while MLV may not provide full or universal protection and do not prevent infection, they contribute to decrease the impact of the disease, thereby reducing both production and economic losses[877].

In gilts and sows, MLV reduce viraemia and limit the development of clinical signs, such as number of abortions, mummified piglets, and stillbirths, to varying extents when they are subsequently challenged or exposed to the virus. It results also in an improved reproductive performance and piglet viability[561,563,878,879]. However, they do not fully protect from vertical transmission[873,879,880].

Booster or recall doses are necessary to maintain immunity over time in sows. Nevertheless, in specific cases, repeated MLV administrations does not necessarily produces an immune boost[881,882]. In some cases, despite undergoing multiple vaccinations, some sows can be seronegative and lack both NAb and relevant virus-specific IFN-γ responses. These sows are commonly referred to as poor- or non-responder sows[610,719,883,884]. Fiers et al.[884] reported a 3.5-4.1% presence of multiple PRRSV-vaccinated ELISA non-responding sows in Belgium, and a 3.3% tested negative to both ELISA and VNT, with no significant differences noted between parities. Additionally, 40% of the 70 PRRSV-vaccinated herds examined had at least one ELISA non-responder out of twenty sampled sows. Furthermore, the presence of these sows was not related to a specific MLV. In subsequent studies, Fiers et al.[885,886] also evaluated the impact of these ELISA non-responding sows on their offspring. The findings indicated that piglets born from such sows lacked MDA, showed an earlier seroconversion upon vaccination, and exhibited a higher earlier viraemia and viral shedding post-challenge compared to piglets born from seropositive sows. Consequently, these animals may play an important role in PRRSV transmission within a farm.

In growing pigs, numerous publications have reported that MLV mitigate clinical signs and lung lesions in variable levels, as well as decrease viraemia duration and viral load[564,856,868,887,888]. This results in reduced virus shedding and transmission, with several studies indicating a reduction of reproduction ratio (R0) value when pigs are vaccinated with MLV[603,634,889,890].

Regarding the safety of MLV, PRRSV-1 MLV can be considered clinically safe[891]. However, with certain MLV there is a potential issue related to viral replication in pigs and subsequent viral shedding and transmission[439,891,892]. Vertical transmission of the vaccine virus may occur when vaccinating pregnant females at late gestation, together with a risk of increased number of stillbirths and mummified piglets[427,879,893]. Furthermore, MLV have been reported to cause viraemia for up to six weeks in pigs[505], which certainly can contribute to the described circulation of MLV within herds and spread to neighbouring herds[669,894–896]. For instance, this has been evidenced with the detection of MLV-derived virus in non-vaccinated pigs, as well as the detection of PRRSV-2-MLV-derived virus in countries where the vaccine is not authorised[695,896–899]. Bálint et al.[900] detected a high frequency of potential PRRSV-1 vaccine-derived circulating strains in Hungary, by

analysing ORF5 and ORF7 sequences, as well as reported that commercial vaccines varied in terms of their genetic stability.

If the MLV remains replicating within the population, it poses a dual risk – there is the potential for reversion to virulence and the occurrence of recombination. In fact, as reviewed later for recombination (see *Section 1.10.3.2. Recombination*), examples of both events have been reported in the field. Regarding virulence reversion, for instance, a PRRSV-2 MLV vaccine used in Danish herds caused increased number of abortions, weak-born and stillborn piglets, and mortality in the nursing period in several herds. This revertant spread to non-vaccinated animals within the vaccinated herds and to non-vaccinated herds. Furthermore, vaccine virus was also transmitted through semen of vaccinated boars to other herds[669,901–903]. Another example has been reported in China by Jiang et al.[904], who identified three field strains derived from an HP-PRRSV-vaccine strain that caused high fever and mortality in piglets. Recently, there have been reports in which potential HP-PRRSV MLV candidates showed reversion through several passages in pigs[905]. Opriessnig et al.[875] identified a possible PRRSV-2 MLV-derived virus as the cause of several outbreaks.

Eclercy et al.[892] showed that after two transmission events of a PRRSV-1 MLV in pigs, the vaccine virus regained virulence resulting in clinical signs and increased viraemia and nasal shedding. This phenomenon was potentially linked to five mutations located in nsp2, ORF3, and ORF5. Additionally, three mutations, two in nsp2 and one in ORF5a, were identified as linked to pig re-adaptation of the MLV.

Furthermore, since MLV do not confer sterilizing immunity, they may promote the selection of viral variants escaping the immune response[848]. Costers et al.[336] reported escape mutants capable of circumventing neutralizing antibodies against GP4. Besides, Nilubol et al.[906] reported that the implementation of a PRRSV-2 MLV in a herd increased the genetic diversity of PRRSV-2 over a one-year period follow up. Kwon et al.[907] observed an expanded genetic distance and diversifying evolution in PRRSV-1 structural proteins, together with the emergence of new glycosylation sites in the neutralizing epitopes of minor structural proteins, following the introduction of PRRSV-1 vaccination in Korea.

1.9.5.2. Inactivated vaccines.

Inactivated vaccines (IV) are deemed safer than MLV as they do not replicate and transmit to other pigs. Despite their safety, IV have a limited efficacy in eliciting a protective immune response when used alone, inducing very low or null titres of NAb and weak CMI responses and providing only weak or null protection upon challenge[560,562,565,855,882,908–910]. Commercially available IV were unable to prevent or reduce viraemia in vaccinated pigs when challenged[562,854]; neither prevent viraemia, vertical transmission, or reproductive failure in gilts[911]. Moreover, when applied in infected animals, IV have been reported not to reduce viral shedding, although there are controversial results[909,912,913].

However, IV boost the NAb and CMI responses of pigs previously primed with the live virus[560,580,855,882]. Furthermore, repeated administration of IV seem to also prime the immune response as well as to boost pre-existing immunity, inducing NAb and CMI responses[562,580,855,909,911]. In fact, systematic vaccination of sows with an IV for eighteen months resulted also in an improved reproductive performance and piglet viability[914].

The efficacy of IV can differ depending on the specific technique used for inactivation and the type of adjuvants incorporated into the vaccine formulation[515,560,910,915].

1.9.5.3. Vaccination strategies against PRRSV.

While vaccines do not provide full and universal protection against PRRSV infection, they can significantly reduce the impact of the disease. However, to ensure efficacy, vaccination should be coupled with stringent biosecurity, control measures, and PRRSV surveillance[916,917]. To guarantee optimal effectiveness, it is crucial also to adhere to vaccine guidelines, including doses, handling, and storage. Moreover, to reduce the risk of recombination, only one MLV should be applied in a herd and, if the vaccine is changed, it is crucial to provide an adequate time gap between administrations, to prevent potential simultaneous infections with both vaccine viruses in the animals.

On farms, a standard practice involves vaccinating breeding sows, and optionally, piglets. When it comes to the breeding population, vaccination protocols must discern

between replacement gilts and sows currently in production. As stated previously, naïve gilts acclimatization is critical to build herd immunity. Different strategies can be used, of which the safest approach is to vaccinate. Based on the described characteristics of each vaccine type[562], MLV vaccines are crucial for an effective initial immunization, while IV are mainly employed for boosting or recall purposes. Thus, widely adopted protocols involve vaccinating gilts first with a MLV and revaccinating them before the introduction to the breeding herd. To verify the effectiveness of vaccination, it is recommended to assess seroconversion using ELISA approximately from two to three weeks after the completion of the immunization protocol.

In the breeding stock, vaccination serves the primary objectives of establishing a consistent immune status, reducing vertical transmission from sows to foetuses, and providing MDA to their offspring. For this purpose, sows are vaccinated at regular intervals to maintain herd immunity and avoid the existence of negative subpopulations. Conventional vaccination strategies include production cycle vaccinations or whole-herd calendar vaccinations. The former one may consist, for instance, of vaccinating sows six days after farrowing and at sixty days of gestation, or at the beginning of gestation and ninety days later. These types of protocols allow for a more consistent level of MDA, leading to a more homogeneous immune status in the offspring, delaying viral circulation, and reducing the impact of PRRS and secondary infections[596,918]. Martín-Valls et al.[595] showed that priming sows with and MLV at week 8 of gestation and re-vaccinating them with an IV three weeks prior to farrowing resulted in the homogenising of levels of antibodies and the reduction of PRRSV incidence in piglets for six weeks.

The whole herd calendar vaccination, also known as blanket vaccination, is the currently most common applied protocol and consists of periodic mass vaccinations of the breeding herd. In other words, all sows are administered recall vaccinations with MLV or IV at the same time, from two to four times per year. While this protocol yields positive reproductive outcomes at the herd level, its drawback lies in the potential negative effects at the individual level, contingent upon the sow's gestational stage at the moment of vaccination. Some authors do not recommend MLV vaccination in pregnant sows, since it may result in an increased number of stillbirths and mummified piglets and a reduced number of born-alive and weaned piglets[893,919]. When MLV was implemented in a naïve herd in mass vaccination, there was a transient increase in mummified piglets and pre-

wean mortality, and a reduction of live born piglets, yet without affecting total pigs weaned[920]. However, Linhares et al.[877] reported that mass vaccination had an impact of one piglet per sow per year, while Moura et al.[921] showed that MLV vaccination in stable farms resulted in even a lower impact in productivity, as pre-weaning mortality increased by 0.26% two weeks after vaccination, while other herd performance parameters remained unaffected. Furthermore, mass vaccination led to a more rapid recovery of production losses and reduced piglet losses in outbreaks compared to immunization by live virus exposure[831].

Mass vaccination has also been used as an emergency measure in PRRSV-outbreaks[916]. The question of whether a herd facing a PRRSV outbreak should receive a MLV vaccination to mitigate the clinical outcome is a matter of ongoing debate. The goal is to induce immunity to those animals that have not been infected yet and reduce the number of susceptible animals, thereby shortening the duration of the outbreak. In infected animals, evidence supports a decrease in the viral shedding of the field virus following vaccination[857,888,922]. However, the simultaneous presence of PRRSV field and vaccine strains in the herd raises concerns about the potential for recombination.

Piglet vaccination may be a useful tool to control PRRSV transmission within the population[634,889]. MDA, acquired through colostrum intake in the first hours post-birth, play a significant role in piglet defence during the early weeks of life. However, these MDA can pose a challenge for vaccination, as they may persist several weeks, depending on the colostral antibody intake[597,598,918].

Despite MDA have been shown to interfere with vaccination[597,598], several reports have proven reduction of viraemia, clinical signs, and improved growth performance when vaccinating two- to four-week-old piglets, even in the presence of MDA[871,872,923,924].

Some vaccines are licensed to be used from day 1 of life to induce immunity as early as possible. Although there were uncertainties about the immune system's ability to respond to vaccination and potential interference by MDA, several studies have reported the development of an immune response and a decrease in viraemia, viral shedding, clinical signs, and lung lesions, coupled with an improved growth performance and reduced mortality, in vaccinated one-day old piglets[925,926]. In fact, a meta-analysis of a PRRSV-2 MLV showed that vaccination at one or twenty-one days of age led to a reduction of viral load, clinical signs, lesions, and mortality[864].

1.9.6. Future strategies to control PRRSV.

A novel approach for controlling PRRSV is gene editing and selective breeding of PRRSV-resistant pigs (further reviewed[927]). Previous studies showed that PRRSV susceptibility or resistance differ between breeds and individuals[391,430,928–931]. Certain Asian local breeds showed some level of resistance against HP-PRRSV, only showing mild signs during infection[932–934]. The focus on ongoing research involves screening and identifying the genetic mechanisms underlying variations in PRRSV susceptibility and resistance. Some of the identified host factors are related with cytokines, PRRSV-cell receptors, SNPs, intestinal microbiota, and non-coding RNA (further reviewed[935]). Recently, a company launched a lineage of CD163 gene-edited pigs that are resistant to PRRSV because they lack the essential viral receptor. This strategy has been proved to be effective in experimental models[96–98,127,936]. Another approach to control the disease is to select and breed pigs or sows resilient to PRRSV[937,938].

Additional innovative strategies involve the development of antiviral molecules aimed at blocking PRRSV entry, employing RNA-based therapeutic strategies, using chemical compounds, and nanobodies for PRRSV inhibition, as well as the application of immune stimulators, among various approaches (further reviewed[939]. For instance, one method is to target and regulate or block CD163 (further reviewed[940].

1.10. PRRSV EVOLUTION AND GENETIC DIVERSITY

1.10.1. Insights into viral evolution.

Viral evolution unfolds both on the infected individual and herd levels. While the latter encompasses the former, they are not exactly equivalent. The circumstances occurring in the individual will dictate the evolution of the virus within each subject, regardless the transmission efficiency of the virus. At the herd level, transmission becomes the key factor. Viral variants unfit to be transmitted will disappear sooner or later, being replaced by variants with better transmission efficiency. However, these levels are intricately interconnected since the variants fittest for transmission will be generated within an infected individual.

RNA viruses are the quintessence of plasticity among animal viruses. Their RdRp, devoid of proof-reading capability, inherently introduces variability during replication, giving rise to what is known as quasi-species. The term quasi-species refers to groups, clouds, or swarms of non-identical yet related viruses existing in a dynamic equilibrium marked by variation, competition, and selection[941–945].

The ongoing expansion of the virus creates highly diverse and dynamic viral populations that can adapt and successfully transmit in diverse environments and to new hosts. Within this dynamic process, selection plays a crucial role by promoting the survival and replication of variants carrying mutations that enhance their ability to adapt to the new environmental challenges, such as those posed by the host's immune system, and have greater transmission and epidemiological fitness[946,947].

Selection, acting as a driving force in viral evolution, results in either positive or negative outcomes. Positive selection occurs when a given mutation or variant is fixed and establishes itself within the population, most often because it confers an adaptive advantage, allowing these variants to thrive. Conversely, negative selection, or purifying selection, operates against deleterious variants, eliminating or maintaining them at a low frequency to preserve essential regions in the viral genome. Additionally, neutral selection can also influence virus evolution by limiting genetic diversification[945,948].

Various forces come into play to influence the selection of the viral populations. The quasi-species, in constant evolution, are shaped by a combination of intrinsic and extrinsic factors. Intrinsic factors include factors that promote viral replication, replication accuracy, transmission, or immune escape. Externally, the host's immune response exerts a selective constraint on the quasi-species, exerting pressure on the viral cloud to generate a fitter virus to escape from it. For example, this may result in the selection of variants escaping to the control of the immune system, with more efficient replication rates, with improved cell tropism, or more efficiently transmitted[945,949]. The mutations will either prevail, persist, or diminish based on the fitness of the mutants - how effectively they adapt to selective pressures and influence the virus's transmission capacity.

Often, a common perception is that the prevailing variant in the quasi-species is the fittest of the fittest. However, this notion is not entirely accurate. Given the continuous introduction of variability, the offspring of the fittest would result in variants less adapted

than the parental. In contrast, the prevailing variants are those characterized by having superior adaptability under certain specific conditions, with its offspring having a higher probability of transmission. This ensures their continued presence and influence within the viral population.

Host-to-host transmission plays an essential role in viral evolution. Without transmission, there is no viral replication, and without replication, there is no viral population. Ultimately, only those viral variants efficiently transmitted between individuals will persist in the population, while those unable to be transmitted or with an inefficient transmission will disappear. Moreover, transmission acts as a fundamental driver of viral evolution, shaping the genetic diversity.

During transmission, a phenomenon known as bottleneck often occurs, limiting the number of viral variants that successfully establish infection in the new host[950–952]. Bottlenecks may involve a reduction in the size of the viral population being transmitted, a diversity constraint of the viral population, or both. Viruses that cross transmission barriers more efficiently are more likely to persist and spread within populations.

Tight bottlenecks, as seen for HIV, influenza, and SARS-CoV2, limit viral evolution by restricting the mutations that are passed on during transmission. In fact, only a limited number of distinct viral genomes of these viruses generated in the host, or even only one donor genotype, are transmitted during infection[952,953]. This filtering effect of transmission bottlenecks plays a crucial role in shaping viral evolution. By limiting diversity, they create a more focused environment for selection. This can also facilitate genetic drift, where random mutations can become fixed through the founder effect. This phenomenon underscores the significant impact of transmission dynamics on the genetic diversity and viral evolution. Beyond transmission among individuals, the transmission between cells and tissues within the same host can introduce unique selection pressures and provide a novel environment to which the virus must adapt, influencing its evolution[948,950,951].

In the context of PRRSV, another crucial factor comes into play—the size of the host population and the way in which pigs are raised. Farms, with their substantial number of animals highly-densely confined, create conditions conducive to the efficient spread of the virus. The greater the size and density of the population, the more pronounced the

potential for transmission. In addition, in modern production systems, replacement rates are very high (often reaching 50% per year in many farms), resulting in a continuous inflow of susceptible animals that serves as a continual source fuelling transmission. Certainly, the transition in pig production practices in the late 20th century towards high-density animal farming has facilitated the transmission and subsequent genetic expansion of the virus[39,954].

Furthermore, within a herd, the levels of immunity among different animals can be diverse, adding an additional layer of complexity to the dynamics of transmission. Sows, boars, or older grower pigs may have developed immunity owing to a previous infection or vaccination, while young piglets may have MDA, or may be naïve after the waning of colostral immunity. This may influence how the virus spreads through the population in different phases. Moreover, in the case of PRRSV, prior immunity - whether from natural infection or vaccination - does not necessarily provide complete protection. Thus, a substantial proportion of infections will happen in partially immune animals. In such cases, when viral circulation persists in the herd, the viral population must overcome the selective pressures exerted by pre-existing immunity.

1.10.2. PRRSV genetic diversity.

Overall, for PRRSV, both the characteristics intrinsic to pig production, as well as those inherent to the virus, collectively create the environment for viral evolution. A hallmark and a major challenge associated with PRRSV is its extremely high and ever-expanding genetic diversity[39,955]. PRRSV genetic diversity primarily arises from mutation, recombination, and selection, resulting in an exceptionally varied array of isolates exhibiting diverse characteristics. This heightened genetic diversity results in significant variations in terms of virulence, antigenicity, and interaction with immune system cells, among other factors.

Notably, PRRSV-1 and -2 emerged independently and nearly simultaneously in two different continents and have been evolving since then. Initially, it was thought that PRRSV-2 displayed greater genetic diversity, whereas PRRSV-1 was more conserved and showed a lower degree of variation[330,956,957]. This perspective shifted when extending the analysis of PRRSV-1, revealing a similar or even greater diversity among PRRSV-1 than

PRRSV-2[34,35,52,668,958–961]. Nonetheless, genetic diversity of PRRSV is continuously expanding for both species[49,573,962–964].

The genetic diversity and molecular evolution of PRRSV, along with their effects, have been previously addressed (further reviewed[38,39,52,277,775,955]). Indeed, numerous publications have analysed and compared genetic diversity and evolution, mainly ORF5-based, both on a geographic scale and over time, identifying mutations and potential N-glycosylation sites of relevance[688,695,773,779,959,962,965–968]. However, little is known regarding how PRRSV diversifies and evolves at both the individual and herd levels over time, and the underlying mechanisms and genetic determinants of this genetic diversity and evolutionary dynamics. In the following sections, we will review what is known about PRRSV.

1.10.3. Key drivers of PRRSV evolution.

1.10.3.1. Mutation.

Mutation has been classically presented as the key driver of PRRSV diversity. It has been estimated that PRRSV has one of the highest mutations rates among RNA viruses, which range from 10^{-3} to 10^{-5} mutations per nucleotide incorporated per replication[31,969,970]. Applying this mutation rate estimation, each virion in the resulting progeny would harbour at least one mutation. This high mutation rate arises from the lack of 3' proofreading capacity of the RdRp[970,971]. True to its nature, PRRSV does not exist as a singular isolate but as a quasi-species within infected pigs, which evolves continuously[378,379,972]. Mistakes during replication can yield mutations that may be deleterious, neutral, or beneficial, contributing to the composition of the quasi-species. Although present in the quasi-species, not all mutations will become predominant.

Several authors attempted to estimate the substitution rates of PRRSV. Hanada et al.[31] provided a range of 4.71 to 9.8 x 10^{-2} substitutions per position per year based on ORF3-5 of both PRRSV-1 and -2 that would make PRRSV the animal virus with the highest substitution rate ever reported. However, these estimations are somewhat conflicting with those of other authors. For example, Forsberg et al.[30] initially calculated a nucleotide substitution rate of 5.8 x 10^{-3} substitutions per position per year based on

PRRSV-1 ORF3 that was later recalculated to 1.8 x 10^{-3} substitutions per position per year[32]. Since then, other authors have also estimated the rate, mainly based on ORF5[54,973]. Yoon et al.[974] reported a 1.55 x 10^{-3} substitutions per position per year rate based on PRRSV-1 and-2 ORF5-7. Pamornchainavakul et al.[975] estimated distinct evolutionary rates for various fragments of PRRSV-2 complete genome. Also, they documented that PRRSV-2 lineages had different rates, a finding consistent with other studies[976,977]. It is worth noting that the substitution rate can be influenced by the transmission dynamics. As a result, obtaining data from different populations with varying selective pressures may result in different estimations.

The gradual accumulation of random mutations in the genome can occur over time, leading to the progressive evolution of the virus[960,961,978]. It has been estimated that PRRSV undergoes a drift of 0.5% - 1% per year based on ORF5 sequences[573,783,963]. Again, these values must be considered as estimations of magnitude since different epidemiological conditions and selective pressures may yield different values.

Accumulation of mutations may be a potential factor influencing changes in virulence. A key event in PRRSV history was the emergence of HP-PRRSV in 2006 in China, which has been suggested to evolve from the Chinese local diversity of PRRSV strains[59,187,979]. Despite initially attributing the virulence to a specific nsp2 deletion, no specific virulence determinants have been identified for this highly pathogenic variant [190]. Certainly, extensive research has been conducted to pinpoint mutations linked to virulence and attenuation, although success in this regard has been limited. It appears that the interplay of multiple genes contributes to these processes and may be strain-specific[238,849,852,979–981]. For instance, studies indicated that nsp9 and nsp10 play a role in the replication efficiency of Chinese HP-PRRSV, contributing to their heightened virulence[238–240].

1.10.3.2. Recombination.

PRRSV genetic diversity has been mostly attributed to the combination of a high mutation rate of the RdRp, long infection periods, and large viral populations. Although all these elements contribute significantly to the diversity of the virus, recombination is also an important driving force for the evolution of PRRSV. Indeed, thorough analyses of

complete genome sequences have revealed frequent recombination events spanning the genomes of both PRRSV species[781,982].

Recombination primarily takes place when a cell is simultaneously coinfected with two or more distinct strains. In positive-sense RNA viruses like PRRSV, this process has been proposed to adhere to a copy-choice or template switching model (Figure 8), particularly during the transcription of subgenomic mRNA, where the RdRp switches templates. This event is more likely to occur in regions characterized by high sequence similarity[983,984].

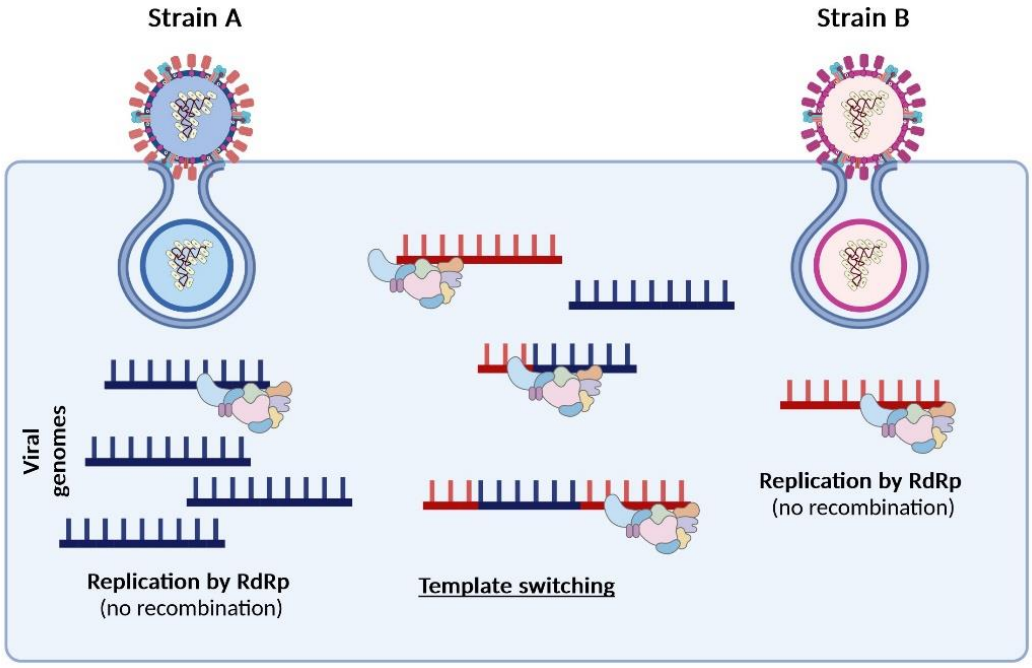

Figure 8. Recombination by template switching. Template switching or copy-choice refers to the process where the RdRp switches from one RNA template to another during replication. The RdRp initiates replication on one viral RNA (B, red) and subsequently "jumps" to the second viral RNA (A, blue), resulting in the creation of a recombinant genome composed of segments from one of the parental strains (A) and segments from the other (B). Created with BioRender.com.

In any case, while a recombination event is necessary to find a recombinant PRRSV isolate on a farm, it is not the only requirement. Recombination may yield both viable or non-viable genomes; consider the case where the recombinant genome undergoes extensive deletions or insertions that made impossible the assembly of a functional virion. Even if a viable recombinant is produced, it needs to gain competitive advantages to persist

and outcompete the parental strains within the herd. Such advantages may be a better or higher receptor affinity, an increased replication capability, or a decreased susceptibility to pre-existing antibodies, among others. In other words, only those recombinant genomes with increased fitness have the potential to persist. Recombinant viruses that have become established, expanded, and gradually replaced other circulating strains in a geographic region have been reported in China and some regions of the United States[61,982,985], reinforcing the importance of recombination in PRRSV evolution and epidemiology. The result of a recombination event in terms of virulence, replication rate, immunogenicity, or capability to escape pre-existing immunity remains unpredictable[986,987], although certain recombinant isolates may result in enhanced pathogenicity, as documented by several studies[61,62,982,986,988–991].

Recombination in PRRSV was first proven *in vitro* by Yuan et al. [992], who generated recombinant viruses by coinfecting MA-104 cells with two distinct PRRSV-2 strains. Early studies estimated *in vitro* a recombination frequency between 0.1-2.5% and 2-10% for PRRSV-1 and PRRSV-2 field isolates, respectively[782,992]. Murtaugh et al.[978] reported that recombination occurred in field samples. In another study, Murtaugh et al. [993] documented recombination between MLV *in vitro*, both in MA-104 cells and PAM, with a frequency of 1/6 in PAM. When experimentally co-infecting animals with two PRRSV-2 field strains, recombinant viruses were generated in four out of five pigs[994]. Since then, recombination has been reported both *in vitro* and *in vivo*, as reviewed later. These recombination events are not limited only among field isolates but also extend to occurrences between vaccines, as well as between field isolates and vaccines or vaccine-like strains. No recombination events have been found between the two PRRSV species and the risk is considered to be low due to the low similarity between them[782].

It is well known that, for positive-stranded RNA viruses, while template switching can potentially occur at any point in the viral genome, the generation of viable recombinants is not equally likely throughout all the genome [995]. These viruses have relatively small genomes (15 kb in PRRSV) with limited tolerance to deletions or insertions without compromising viral viability. The existence of hotspots in the PRRSV genome remains unclear, since breakpoints have been identified in several sections of the viral genome, involving both structural and non-structural proteins. It has been widely assumed that recombination breakpoints might be associated with body transcription

regulatory sequence (TRS) elements, as they mediate sgmRNA synthesis, or that certain RNA secondary structures might be in proximity to the breakpoints[27,39,774,996]. However, evidence supporting the involvement of these elements in PRRSV recombination is lacking.

Early studies found indication that recombination in PRRSV occurred in ORFs 2, 3, 4, 5, and 7, but not ORF6, yet the sample was limited to 10 PRRSV strains[997]. Other reports analysing genetic diversity and evolution of PRRSV-2 over time have shown that recombination breakpoints, although widely distributed, localize with higher frequency in nsp7, nsp9, and ORF2-4[976,998,999]. Particularly, Yu et al.[998] documented the potential existence of inter-lineage recombination hotspots, defined as genomic regions with frequency higher than 25%. These hotspots would be localized in nsp9 and ORF2-3 for type 2 PRRSV, while intra-lineage recombination varied greatly with hotspots in nsp1-nsp2, nsp4, nsp6-10, ORF2-3, and M for PRRSV2. Certainly, Cui et al.[999], who also reported that many breakpoints concentrated in nsp9, showed that artificially constructed recombinant variants with this segment switched acquired a higher replication in PAM and MARC145-cell cultures, in agreement with the introduction of a more efficient RdRp. Additionally, there are also examples of recombination between HP-PRRSV and MLV-derived strains, in which the recombinant part covered both nsp9 and nsp10 and resulted in higher replication rates and virulence[988,1000].

For PRRSV-1, recombination breakpoints appear to be also scattered randomly throughout the genome as for PRRSV-2. Vandenbussche et al.[774] documented higher recombination frequencies in the structural proteins and identified potential hotspots in specific regions, namely nsp1, nsp7α, nsp12, ORF2-5, and M-N regions, based on the analysis of 125 PRRSV-1 recombination events. The authors also observed a tendency for recombination breakpoints to take place within the 10% beginning and endings of genes.

Considering the high prevalence of PRRSV infected farms and pigs in endemic areas, concomitant infections with various strains can be frequent, creating an optimal environment for recombination to occur. Co-infection of a pig by more than one strain, even with both PRRSV-1 and -2 strains, has been previously reported[774,906,1001,1002], as well as co-circulation of different strains in a farm[660,711,778]. In fact, Lalonde et al.[773], through WGS, reported a 4.55% prevalence of coinfections in pig herds in Canada, and a 6.52% of recombinant variants among WGS PRRSV strains. Additionally, Trevisan et al.[791] found at

least two co-circulating strains in 18/20 followed farms and in 8/20 one of the viruses was a vaccine-like strain. According to the same authors herds with three or more co-circulating strains had a greater likelihood of recombination[791]. Furthermore, repeated sampling in two herds revealed the continued circulation of the recombinant strains[774], suggesting that some of them may become resident in the farm. It is worth noting that due to the mechanism in which recombination is based, this is a probabilistic event. The higher the copies of genomic RNA of each parental strain within the cell, the greater the likelihood of the recombination event. Likewise, the longer the period of infection by one strain, the higher the chances of being infected by another one. In light of this, vaccinating animals already infected with a MLV increases the chances of co-infection and occurrence of effective recombination. Accordingly, it can be deemed a high-risk practice.

The following sections will provide a brief review of the importance of recombinant isolates in PRRSV epidemiology.

1.10.3.2.1. Epidemiological importance of recombination in PRRSV-2.

Before the emergence of HP-PRRSV in China only a few recombinant viruses had been identified in that country[780,999,1003]. However, it should be noted that this scarcity may be attributed to incomplete data, possibly due to the lower use of whole-genome sequencing in the past. Since the emergence of HP-PRRSV in China, there has been a concurrent circulation of the classical PRRSV-2, HP-PRRSV, and, to a lesser extent, PRRSV-1. This is coupled with the extensive use of up to seven distinct MLVs derived from classical PRRSV-2 or HP-PRRSV[965,999]. This scenario, along with the high replication rate and transmission of HP-PRRSV and the large pig population in the country, has created an environment conducive to recombination in the field. In fact, recombination events have been implicated in major outbreaks, such as the 2009-2010 and 2013-2014 outbreaks attributed to recombination between HP-PRRSV strains[982,986–988,1000] and between the NADC30-like and HP-PRRSV strains[61,1004,1005], respectively. The introduction of NADC30-like PRRSV from North America in 2013 heightened recombination frequency, leading to widespread outbreaks in subsequent years[62,1006–1010]. Additionally, the emergence of NADC34-like PRRSV in 2020 has further contributed to the genetic diversity through recombination with other PRRSV-2 strains[990,991,1011,1012].

Recombination events involving MLV vaccines, HP-PRRSV, and classical PRRSV-2 continue to be reported, with increasingly complex patterns over time, including triple and quadruple recombinants between various lineages[1013,1014989,990,1008,1011,1015–1017]. The continuous recombination events contribute to the evolving genetic diversity and complexity of PRRSV in China.

Recombinant viruses between different lineages have also been circulating in United States, Canada, Mexico, and Korea[773,792,985,1018,1019]. Notably, from 2020 to 2021, a fast-spreading PRRSV-2 variant emerged and caused unprecedented production losses in United States[63]. This emerging variant was found to be an intra-lineage recombinant virus between several L1 sub-lineages[975]. Trevisan et al.[791] found recombination events in 12/20 followed farms, ten of which were between field strains and two between field isolates and MLV strains. Furthermore, several recombinant variants between field isolates and MLV strains have been also reported[64,1018,1020].

It has been shown that the recombination rate, both inter- and intra-lineage, recombination pattern and complexity in relation to the parental strains varies among lineages and sub-lineages, as well as geographically and over time[965,976,999]. Furthermore, recombination rate is associated to high pig density[999].

1.10.3.2.2. Recombination in PRRSV-1.

Recombination among circulating field strains has also been documented for PRRSV-1 in Europe, China, and the United States, and is also a common occurrence[49,774,781,958,1021–1023]. Noteworthy, a highly virulent PRRSV-1 strain, resulting from various recombination events, recently emerged, and has led to severe PRRSV-1 outbreaks in North-eastern Spain since 2020[70,71]. These outbreaks occurred regardless of farms implementing vaccination or being PRRSV positive. Furthermore, during the investigation of several of these outbreaks, an additional recombinant event involving a vaccine strain was detected[71]. This certainly emphasizes the substantial impact that recombination can have.

Instances of recombination between field strains and vaccines in PRRSV-1 have also been observed, with some recombinants being implicated in outbreaks. The first report in Europe was in Britain, involving a recombination event between a field strain and the

vaccine in use at that time[964]. In Hungary, a vaccine-derived recombinant was identified in a surveillance[1024]. Vandenbussche et al.[774] found that a vaccine or vaccine-like strain was involved in 15 out of 125 analysed recombinant events of PRRSV-1, with all four commercially available vaccines in Europe being implicated. Furthermore, multiple mosaic strains with traces of multiple vaccine strains were also identified[774]. Recently, two PRRSV-1 isolates from geographically distant farms with reproductive or respiratory clinical outbreaks from Austria and one from Germany were found to be originated from the recombination of a field strain and a vaccine[996]. In China, Chen et al.[1025] identified also a recombinant between a vaccine and a field strain.

1.10.3.2.3. Recombination between vaccines.

As mentioned earlier, there are also several reports of recombination between MLV or MLV-like strains. In France, a recombinant virus between PRRSV-1 MLV Unistrain™ PRRS and Porcilis™ PRRS was found in a farm where both MLV were used successively[1026]. This virus persisted circulating in the farm at least for two years, with a 1.4% genetic divergence. The recombinant strain showed an increased excretion and transmission capabilities compared to the parental vaccine strains in experimentally infected animals[1026,1027].

In 2019, in Denmark, several outbreaks linked to the same boar station were caused by a recombinant virus between MLVs Unistrain™ PRRS and Suvaxyn™ PRRS[1028]. The recombinant virus was highly transmissible and more virulent compared to the parental strains, resulting in a significant decrease in productivity during the subsequent five months[1028,1029]. However, under experimental conditions, the strain was shown to have a comparable, or exceeding, pathogenicity than typical PRRSV-1 subtype 1 strains, yet less virulent than subtype 2 and 3 strains[1030,1031].

In China, a recombination strain between PRRSV-1 MLVs Unistrain™ PRRS and Porcillis™ PRRS have also been reported[1032]. Regarding PRRSV-2 MLVs, Zhang et al.[1033] found four different recombinant strains continuedly isolated from a single farm, one of which was a recombinant virus between MLVs TJM-92 and HuN4-F112 and another one between two MLV-like viruses evolved from HuN4-F112.

Trevisan et al.[1034] identified a recombinant variant between two PRRSV-2 MLV, specifically Ingelvac™ PRRS MLV and Prevacent™ PRRS, in a wean-to-finish in the United States. The observed symptoms included coughing and increased mortality. The piglets had received vaccination with Prevacent™ PRRS, while the breeding females were vaccinated with Ingelvac™ PRRS.

All these findings emphasize the need for implementing good vaccination practices directed to reduce the probability of recombination events involving vaccine viruses.

1.10.3.3. Selection.

Within the quasi-species framework, PRRSV is continuously adapting and evolving in response to selective pressures and challenges[379,1035]. When genetic variability has been examined during a PRRSV infection, it has been observed that new variants emerge over time[369,378,379,1036]. Despite these observations, the specific selection pressures and the underlying mechanisms leading to their emergence remain elusive[331,378,960,972,1036]. Further elucidating these aspects would contribute significantly to our understanding of PRRSV dynamics and evolution.

As previously discussed, selection involves a complex interplay of both intrinsic and extrinsic factors. Among intrinsic factors, the transmission capacity emerges as a critical determinant for overall success at a population level. The specific mechanisms contributing to enhanced transmission may be diverse, yet the virus's replication efficiency stands out as a potential key player. If a variant can replicate faster or to higher yields within a given context, it gains a competitive edge and is more likely to succeed, particularly if this translates into an increased viral shedding that can facilitate transmission [628,675]. In the context of PRRSV, differences in replication efficiency have been documented among strains[238–240,366,372]. Oleksiwicz et al.[331] observed that the faster evolution of an ORF3 mutant in Denmark was not related to increased selective pressures and suggested it could be related to differences in replication.

The host's innate and adaptive immunity exert diversifying selection, shaping viral evolution. In PRRSV, the immunity, whether derived from prior infections or vaccination, probably exerts a selective pressure on the circulating virus. This force could drive the selection of mutations that enable escape from neutralization or from the cell-mediated

immunity. Also, mutations resulting in a stronger downregulation of the host's innate immune response would be advantageous. However, the contribution of the immune mechanisms to PRRSV evolution remains elusive to date.

The fact that pigs are infected by PRRSV for prolonged periods infection may increase the probability of escape mutant emergence. The on-going diversity within the quasi-species, in turn, may contribute to the extended infection period of PRRSV[379]. However, to date, only GP4 escape mutants have been generated under experimental conditions. These emerged under the pressure of neutralizing antibodies targeting the GP4 neutralization epitope, denoting their potential significance in PRRSV evolution[293,336].

In a study conducted by Cortey et al.[789], the PRRSV quasi-species was analysed and compared between vaccinated animals that were infected and developed short and long viraemia, which corresponded to higher and lower levels of neutralizing antibodies, respectively. In animals with short viraemia, most selected changes were observed in nsp9, GP2, and M proteins, with the latter two being potentially implicated in immune escape. The quasi-species composition of animals with longer viraemia resembled that of the initial inoculum, suggesting that no selection of mutations occurred because of the low levels of neutralizing antibodies. The results underlined the importance of neutralizing antibodies in shaping the genetic diversity of PRRSV. Chen et al.[1037] attempted to analyse the role of immunity in the evolution of the quasi-species, with an experimental challenge of animals suffering a severe combined immunodeficiency and normal animals followed for twenty-one days. However, the same amino acid substitutions were found in both animals, probably being the result of viral adaptation to the host. It is worth noting that most studies indicate that neutralizing antibodies against PRRSV do not develop before the fourth week of infection[498,565]. In another study, Chen et al.[1038] observed a significantly higher number of substitutions during infection rebounds compared to acute infections, pointing to the influence of adaptive immunity as a selective pressure. Their study identified positively selected positions, possibly contributing to immune evasion and viral persistence. Similarly, other authors have also identified regions or positions potentially under positive selection, such as in ORF2, ORF4, ORF5, and ORF6, suggesting susceptibility to immune-driven selection pressures[31,976,1039]. Hypervariable regions and unequal distribution of genetic variability throughout the genome denote that there are regions in the genome that

may be under stronger immune selective pressures than others. Highly variable regions have been identified in nsp1, nsp2, ORF3, ORF4, and ORF5[46,235,369,774,1038].

Mutations may result to changes in the N-glycosylation patterns as well. Numerous studies propose the involvement of GP5 glycosylation in evading neutralizing antibody responses through glycan shielding, diminishing antibody neutralization capacity[290,294,371,1040]. These changes have also been observed during prolonged infections [378,1036] and *in vitro* studies[379]. Acquisition and loss of potential N-glycosylation sites have been analysed in PRRSV ORF5 sequences[572,573,963,964,1039,1041]. Vu et al.[295] found that glycosylations in GP3 and GP5 were important for evading neutralisation response. Furthermore, Wei et al.[1042] reported that animals infected with a mutant lacking all N-glycosylations of GP5 did not develop viraemia or had an antibody response, highlighting their role in replication *in vivo.* In contrast, glycosylations of the GP2 and GP4 may not play a significant role in immune evasion[295,324,325].

Some studies attempted to relate the phylodynamics of PRRSV at a regional level with the selection of specific variants or strains. For instance, analysis of lineage circulation in the United States reveals temporal dynamics, in which these lineages emerge, fade away, and are replaced by others every 8-10 years[967,1035,1043]. This pattern suggests that when a lineage establishes itself in an area, its transmission increases until immunity develops, leading then to a decline in its circulation. The emergence of another lineage, less recognized by the immune system and with higher transmissibility, follows, outcompeting their counterparts[955]. The genetic diversity has been observed to decrease, as a certain sub-lineage becomes the predominant strain before an epidemic[976]. On the other hand, Kwon et al.[907] observed an increase in genetic diversity of PRRSV in South Korea following the introduction of vaccination in the country.

1.10.3.3.1. Transmission bottlenecks in PRRSV.

Research on the existence and potential impact of bottlenecks in PRRSV is limited. In the context of vertical transmission, Rowland et al.[418] observed that, following the experimental infection of pregnant sows, a non-synonymous mutation at position 77 in the ORF5 gene was detected in 60% of the sequenced clones from one sow. This mutation was present in variable proportions ranging from 25% to 100% in her infected foetuses. This

finding hints at the presence of a selection force influencing the viral population transmitted from the mother to the foetus, suggesting that transplacental transmission could be a potential contributor to the genetic diversity for PRRSV. In another study, 111 sows were infected, and ORF5 sequencing was performed, at day 6 post-infection from their sera, and at day 21 post-infection from one of their foetuses[414]. Among the twenty different nucleotide substitutions, nine were non-synonymous, with three of them identified in multiple foetuses. Only in three instances was the mutation shared between the mother and foetus, suggesting the influence of vertical transmission to an increase in genetic diversity. However, this could be also partially attributed to the time lapse between sample collections.

Concerning bottlenecks in horizontal transmission, Cortey et al.[789] observed a consistent reduction in the viral quasi-species diversity of the founder variants compared to the transmissible population after both intranasal inoculation and natural transmission events. Subsequenlty, in most pigs, genetic diversity increased as expected for naïve animals. Moreover, the distribution of preferred and un-preferred transmitted variants in natural transmission events was non-random across the genome; instead, they concentrated in nsp2, nsp4, nsp9, nsp10, and ORF5.

In another study, Eclercy et al.[892] observed a reduction of vaccine viral variants in animals infected by contact from vaccinated counterparts, suggesting that there was a reduction of diversity in the host-to-host transmission. However, sequenced samples were not of the beginning of viraemia; instead, those obtained at the peak of viraemia were sequenced (after fourteen to twenty-four days of contact with vaccinated pigs), so other phenomena could be involved in this diversity reduction.

Differences in quasi-species composition have been documented in blood and tonsils of infected pigs[1038], suggesting the existence of a possible organ- or tissue-based selection. Lu et al.[772] observed that, three days post-inoculation, different single nucleotide variants with varying frequencies appeared in the quasi-species obtained from lungs and lymph nodes of an infected pig. Since the development of an immune response requires more time, this observation could be indicative of some form of tissue-specific selection or bottleneck.

1.10.4. Genetic diversity within the herds.

The genetic diversity of PRRSV within herds can vary significantly over time[35,658,660,964,1044]. An arbitrary threshold of ORF5 nucleotide identity difference of >2-4% is usually used to differentiate strains and determine a lateral introduction into a herd[783]. However, Goldberg et al.[972] documented around 3% genetic variability of ORF5 quasi-species within-animal and within-farm when examining two farms. Co-circulation of more than one strain has also been reported within herds[35,660,711,776,972,1044].

At the farm level, the genetic diversity of PRRSV can arise from the introduction of new variants or the evolution of existing ones[668,1044]. In regions characterized by high livestock density, the frequent movement of animals and the proximity between farms contribute to frequent lateral introductions. For instance, Angulo et al.[1045] reported that lateral introductions represented 42% of the virus detections in wean-to-finish farms in pig-dense regions of United States Midwest. Furthermore, when a lateral introduction occurs and causes a clinical outbreak, it enhances potential mutation and recombination.

When Pesente et al.[668] examined PRRSV variability among twenty-seven PRRSV-positive herds in Northern Italy, they observed that, in the examined context, the introduction of infected pigs was the main source of genetic diversity in herds, rather than the evolution of the circulating strains. This is in accordance with Larochelle et al.[658], who analysed 226 field cases from 174 PRRSV-infected farms over a 4-year period in Quebec, highlighting lateral introductions, particularly through the introduction of infected animals, as a common cause of genetic variability in herds. Their findings also indicated that PRRSV could persist in farrow-to-finish farms for up to 3.5 years, with a 2% annual drift in ORF5. Additionally, they noted that 24% of farms had multiple circulating strains (2-8) either in the same timepoint, or over a period from four months to three years. In 78% of the farms with multiple submissions over time (38% of the total), lateral introductions occurred in less than a one-year interval causing recrudescence of clinical signs. Certainly, the frequency of lateral introductions may vary significantly with different standards of external biosecurity.

The evolutionary dynamics also vary between herds. Goldberg et al.[660] observed differences in the rate of genetic evolution among herds. Although the specific causes were not known, they suggested that variations in herd management practices could be

influencing these differences. Greiser-Wilke et al.[1044] examined ORF5 sequences in eighteen PRRSV-infected herds in Northern Germany at two timepoints, spaced two years apart. Results revealed that in some herds (28%), the infection persisted with and identical or nearly identical strain (>98% similarity), while others were infected by new strains. PRRSV remained detectable in 56% of the herds, with two of them still reporting clinical signs. In one of these two cases, the same strain was circulating. Stadejek et al.[35], when examining the evolution of PRRSV-1 over time on thirteen farms in the Russian Federation, noted the circulation of very closely related strains on most farms over time, with one persisting up to eleven years on a farm. Hence, the results indicate that in endemic farms with no lateral introductions, the virus may persist for long periods of time and, consequently, adaptation to the endemic situation must occur.

Some herds have been subject to follow-up investigations. Dee et al.[778] monitored an endemically infected a farm for sixteen months, detecting virus circulation after herd closure and partial depopulation. Later, lateral introduction of new strains led to new outbreaks and the coexistence of different isolates. Kim et al.[1046] followed a PRRSV-2 endemically infected farm and noted that the entry of PRRSV-1 affected first the farrowing units and rapidly gained predominance over the PRRSV-2 strain. Nilubol et al.[906] reported an increased genetic diversity of PRRSV-2 but observed no impact on PRRSV-1 after implementing vaccination in a mixed-infected herd with both PRRSV-1 and PRRSV-2. A later introduction of a HP-PRRSV on the same farm did not impact PRRSV-1, but led to variations in PRRSV-2 cluster predominance, substitution rates, and recombination events[1047]. By the end of the study, the vaccine-like cluster became dominant and replaced other clusters in the herds.

Kikuti et al.[1048] followed and characterized the genetic diversity in piglets from twenty litters in a PRRSV-naïve farrow-to-wean farm where, shortly after a PRRSV-2 outbreak (10 days), a mass viral exposure with live virus inoculation was implemented. At 3-5 of age, the minimum nucleotide identity for ORF2-7 was 99.84%, decreasing to a 99.57% at 17-19 days of age. The within-animal percent identity between samplings at 3-5 days and 17-19 days of age was ≥99.7% for ORF2-7, while for ORF4 97.3%. Regions with increased nucleotide and amino acid diversity were identified in ORF2, ORF2b, and ORF4-7. Although all sows received the same inoculum, some litters displayed more genetic diversity than others.

Understanding PRRSV's capacity for evolution and the resulting genetic diversity within herds and individual pigs remains a critical challenge. Future research should prioritize whole-genome-based longitudinal studies encompassing both within-animal and within-farm dynamics. This deeper insight into PRRSV evolution would contribute to the development of more effective vaccines and targeted control measures, ultimately leading to enhanced management and control of this disease.

1.10.5. Consequences of PRRSV high genetic diversity and evolution.

PRRSV ongoing evolution and extensive genetic diversity has a significant impact on various aspects, including the emergence of new strains, virulence, diagnostics, immune response, and the overall epidemiological landscape.

As evidenced in previous sections, both mutations and recombination may lead to the emergence of novel strains, including virulent strains. Unfortunately, it is impossible to predict the virulence and impact of the newly emerged strains. Frydas et al.[633] reported that even when two viruses exhibited only an eight-amino acid difference across the genome, it resulted in distinct clinical manifestations and shedding patterns.

The diversity of PRRSV also poses challenges in diagnostics. The limited conservation in the PRRSV genome complicates PCR design. Mutation in the primer binding sites may lead to false negative results, negatively impacting assay sensitivity[35,668,695,696,968,1049]. In addition, some widely used commercial ELISA may yield individual false negatives, particularly when analysing antibodies against PRRSV-1 subtypes II-IV[52]. Furthermore, the traditional ORF5 sequencing may lead to misclassification of recombinant strains and affect the practical interpretation, for instance, when assessing if a circulating strain is related or not to previous strains in a herd or to the vaccine strain. Ideally, whole-genome sequencing should be performed[774].

Moreover, the high and ever-expanding genetic diversity grants PRRSV the capability to elude the immune response and diminish vaccine efficacy. In fact, it contributes to the partial or lack of protection against reinfection in previously infected pigs, together with the failure of full protection of the vaccines (further reviewed[277,393,841–843]).

Furthermore, PRRSV genetic diversity is suggested to contribute to the persistence in herds for long periods of time[369,972]. In turn, persistence in herds involves a higher probability of being infected by more than one strain over time, leading to a potential higher frequency of recombination events. Herd management and biosecurity measures are essential to minimize introduction and spread of the new virus and avoid to subsequently become established in the population. Besides, the mechanisms driving persistence and generation of genetic diversity within herds still elude our understanding.

Chapter 2.

Background, hypothesis, and objectives

Background

Following the introduction of PRRSV into a breeding farm, the infection tends to evolve towards becoming endemic. In the absence of effective control measures, the virus is maintained in the population through the inflow of susceptible breeders, or sows that have lost previous immunity, leading to vertical transmission of the virus to their offspring. Subsequently, infected piglets then carry the infection to nurseries, where most animals become infected after the waning of maternally-derived antibodies. Nurseries may act as potential sources of virus spread to other sections of the farm through various means, such as fomites and personnel.

In most farms, various strategies are employed to control PRRSV, encompassing the vaccination of sows and, eventually, piglets. Additional control measures include the management of the pig flow and the improvement of internal biosecurity. However, despite the implementation of such measures, certain farms fail to achieve stabilization and persistently remain infected endemically for years, even with multiple-vaccinated sows. Some of these farms experience periodic outbreaks.

In those persistently infected farms, two different patterns are observed. The first entails the sustained presence of the same viral strain over time. In cases where no evident failures in internal biosecurity or pig flow management are identified, the emergence of an escape variant is a common explanation. Although there is some indirect evidence supporting this, it has not been clearly proven under field conditions to our knowledge. The second pattern results from the lateral introduction of a new PRRSV strain, probably due to a breach in external biosecurity. In such instances, the resurgence of the disease can be easily explained by the genetic and antigenic diversity of the virus, coupled with decreased cross-immunity between strains.

In general, several factors contribute to the emergence of new virus strains, including the mutation rate and selection pressures, among others, with immunity usually playing a key role. In the context of PRRSV, the expansion of the viral cloud can occur through four main scenarios: a) transmission to newly introduced gilts with either prior or no immunity; b) vertical transmission, where previous immunity can be critical in vaccinated farms; c) horizontal transmission from infected piglets to their pen mates with

maternal immunity; and, d) horizontal transmission in nurseries and fattening units to animals that have lost their maternal immunity.

Gaining knowledge of the mechanisms governing PRRSV evolution in endemically infected farms may shed light on why certain farms become persistently infected and contribute to the development of more effective control strategies.

Hypotheses

1. Viral diversity depends on the size of the viral cloud. As nurseries usually exhibit the highest incidence and a larger population of infected animals on the farm, coupled with the prolonged viraemia in piglets compared to older animals, the majority of PRRSV genetic diversity within the farm may arise from this particular group of animals.
2. Vertical transmission is key for the persistence of PRRSV in endemic farms. In other viral models, vertical transmission is associated to founder variant effects. In the context of PRRSV, it is plausible that these founder effects are also observable.
3. In most viral infections, host immunity is a major driver of virus evolution. Consequently, in cases where escape variants emerge in endemic farms, those variants are anticipated to overcome and evade the pre-existing immunity in the herd.

Objectives

The general objective of this PhD dissertation is to study the generation of genetic diversity and the evolutionary dynamics of PRRSV over time, within the farrowing units and nurseries of farms that are both endemically infected and vaccinated. To achieve this general objective, the specific objectives are:

1. To determine how viral diversity is generated and evolves immediately after the onset of a PRRSV outbreak in a vaccinated herd.
2. To analyse the evolution of PRRSV in a long-term endemically infected farm and characterize the features of the circulating virus at different time points.
3. To examine the evolutionary events of PRRSV in an endemic vaccinated farm approaching stabilization.

PART II:

Studies

Chapter 3.

Study 1

3. STUDY 1

Infection Dynamics, Transmission, and Evolution after and outbreak of Porcine Reproductive and Respiratory Syndrome Virus.

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3.1. ABSTRACT

The present study was aimed at describing the infection dynamics, transmission, and evolution of porcine reproductive and respiratory syndrome virus (PRRSV) after an outbreak in a 300-sow farrow-to-wean farm that was implementing a vaccination program. Three subsequent batches of piglets (9–11 litters/batch) were followed 1.5 (Batch 1), 8 (Batch 2), and 12 months after (Batch 3) from birth to 9 weeks of age. The RT-qPCR analysis showed that shortly after the outbreak (Batch 1), one third of sows were delivering infected piglets and the cumulative incidence reached 80% by 9 weeks of age. In contrast, in Batch 2, only 10% animals in total got infected in the same period. In Batch 3, 60% litters had born-infected animals and cumulative incidence rose to 78%. Higher viral genetic diversity was observed in Batch 1, with 4 viral clades circulating, of which 3 could be traced to vertical transmission events, suggesting the existence of founder viral variants. In Batch 3 though only one variant was found, distinguishable from those circulating previously, suggesting that a selection process had occurred. ELISA antibodies at 2 weeks of age were significantly higher in Batch 1 and 3 compared to Batch 2, while low levels of neutralizing antibodies were detected in either piglets or sows in all batches. In addition, some sows present in Batch 1 and 3 delivered infected piglets twice, and the offspring were devoid of neutralizing antibodies at 2 weeks of age. These results suggest that a high viral diversity was featured at the initial outbreak followed by a phase of limited circulation, but subsequently an escape variant emerged in the population causing a rebound of vertical transmission. The presence of unresponsive sows that had vertical transmission events could have contributed to the transmission. Moreover, the records of contacts between animals and the phylogenetic analyses allowed to trace back 87 and 47% of the transmission chains in Batch 1 and 3, respectively. Most animals transmitted the infection to 1–3 pen-mates, but super-spreaders were also identified. One animal that was born-viremic and persisted as viremic for the whole study period did not contribute to transmission.

3.2. INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the major pathogens of swine. After its emergence in the 1980 decade, the virus disseminated worldwide and, nowadays, this infection has become endemic in most pig-producing countries. The impact of the infection is variable depending on the virulence of the strain and on the presence of other concomitant agents [reviewed in Saade et al. (2020) and Zhao et al. (2021)] but, in general, the costs associated with the disease are significant for the affected farms (Nieuwenhuis et al., 2012; Holtkamp et al., 2013; Renken et al., 2021).

PRRSV is an enveloped, positive single-stranded RNA virus that belongs to the genus *Betaarterivirus*, family *Arteriviridae*, order *Nidovirales* (Brinton et al., 2021). The 15 kb genome encodes for at least 10 open reading frames (ORFs). ORF1a and ORF1b encode for the non-structural proteins (nsp), including the RNA-dependent RNA polymerase (nsp9). ORF2-4 encode for the minor envelope proteins (E, GP2, GP3, and GP4), ORF5 for the major envelope glycoprotein (GP5) and ORF5a protein, ORF6 for the membrane protein (M), and ORF7 for the viral nucleocapsid protein (N) (Snijder and Meulenberg, 1998).

At present, two species of PRRSV are recognized, that had been classified as genotypes until the last taxonomy modification in 2021. *Betaarterivirus suid 1* or PRRSV-1 (formerly called the European genotype) was first identified in the Netherlands in early 1990s (Wensvoort et al., 1991), and *Betaarterivirus suid 2* or PRRSV-2 (formerly designated as the North American genotype) was identified in the United States in 1992 (Benfield et al., 1992).

PRRSV infection causes reproductive failure in pregnant sows and respiratory problems, poor growth rate, and mortality in piglets. After the contact with the virus, susceptible animals develop viremia, that can last from days to months depending on the age; the younger the longer. After the cessation of the viremia, infection persists in lymph nodes for several weeks but finally the infection is cleared. Recovered animals develop strong immunity against the homologous challenge for some months. In contrast, immunity against the heterologous challenge is only partial and infection can take place, although the course is often less severe [revised by Mateu and Diaz (2008)].

The control of PRRSV infection can be achieved by a combination of monitoring, herd management measures, biosecurity programs, and vaccination. Commercial vaccines against PRRSV are made either of attenuated live virus, with or without an adjuvant, or inactivated virus (always with adjuvant). Attenuated vaccines are preferred for the initial immunization of sows while inactivated vaccines are mostly used as recall antigens. In any case, vaccinated animals can be infected since protection is only partial. In sows, the most common vaccination programs start before the first insemination with the aim of protecting them before the first gestation. Afterwards, sows need to be revaccinated every 3–4 months.

Very soon after the discovery of the virus it was evident that PRRSV diversified with a high evolutionary rate. Thus, Forsberg et al. (2001) proposed that, using the ORF3 to set up a molecular clock, the substitution rate was in the range of $4.8 \pm 6.9 \times 10{-}3$ per site per year, a value higher than the estimated one for other nidoviruses, for example coronaviruses (Duchene et al., 2020; Worobey et al., 2020; Ghafari et al., 2022; Tay et al., 2022). This, together with the increasing evidence obtained by sequencing, led to the idea of the ever-expanding diversity of PRRSV (Murtaugh et al., 2010; Shi et al., 2010). In addition, the existence of recombination events further boosts the diversification of the virus (Brar et al., 2014; Martín-Valls et al., 2014).

There are multiple consequences of this genetic diversity. On the one hand, it is known that genetic diversity has an impact on protection. From early studies it was evident that heterologous protection was only partial (Mengeling et al., 2003; Labarque et al., 2004). For example, most strains, induce neutralizing antibodies (Nab) that only significantly neutralize the strain that induced them (Martínez-Lobo et al., 2011). On the other hand, genetic diversity also affects the recognition of T-cell epitopes (Vashisht et al., 2008; Díaz et al., 2009; Parida et al., 2012), and has been related to the immunobiological properties of the strains such as the induced pattern of cytokines (Darwich et al., 2011). Besides this, recombination events have been linked to the emergence of more virulent strains (Shi et al., 2013; Chen et al., 2018; Cui et al., 2022). Moreover, increased genetic diversity and novel glycosylation sites within neutralizing epitopes was observed when vaccination was introduced in a herd (Kwon et al., 2019).

Although the high number of papers dealing with the genetic diversity of the virus, very few have examined how this diversity is generated in the affected farms. It is well

known that once the farm is infected, PRRSV most likely will become endemic [reviewed by Pileri and Mateu (2016)]. However, in areas of high density of pig farms, lateral introductions of new strains may happen every few months because of the widespread presence of the virus and the multiple sources of infection. Tousignant et al. (2015) showed that in the United States about one third of the farms suffered a new infection every year. Thus, one of the most common situations is repeated introductions of different PRRSV strains in farms even though sows are vaccinated. This scenario is ideal for the selection of new variants or for the occurrence of recombination events. Within the farm, viral selection and diversification could happen mainly: (1) in the sows, the ones which maintain the infection cycle in the farm through vertical transmission to the offspring, (2) in the piglets, which are infected as soon as they lose the maternally derived antibodies, or (3) in both age groups. This is an almost unexplored area.

The present study explores the genetic diversification of PRRSV over time from the ending of a reproductive disease outbreak until 11 months later. We examined the generation of founder viral variants, tracked transmission chains, assessed the persistence of the variants in the farm, and evaluated the role of the animals born viremic in the evolution of the virus. The characteristics of different viral variants with regards to replication kinetics and the susceptibility to neutralization were also determined.

3.3. MATERIALS AND METHODS

3.3.1. Case farm and timeline.

The examined farm housed 300 breeding sows and raised their offspring until 9 weeks of age, when they were sent to a fattening unit until market-weight age. The farm had been positive to PRRSV for at least 4 years prior to the start of the study. The farm purchased replacement gilts (young sows that have not farrowed or given birth to a litter yet) from a PRRSV-negative herd. Gilts were vaccinated with a modified live PRRSV vaccine (Porcilis® PRRS, MSD, Spain) at least twice before the first gestation and then, together with all sows, received recall doses at least three times per year (blanket vaccination). With this vaccination program the farm had been stabilized and no clinical signs of PRRS were observed for 2 years until a reproductive outbreak occurred. The disease was characterized by abortions, stillbirths, and weak-born animals. PRRS diagnosis

was performed by RT-qPCR, but the viral isolate was different from the one circulating before, indicating a lateral introduction of the virus. The timeline and design of the study is shown in Figure 1.

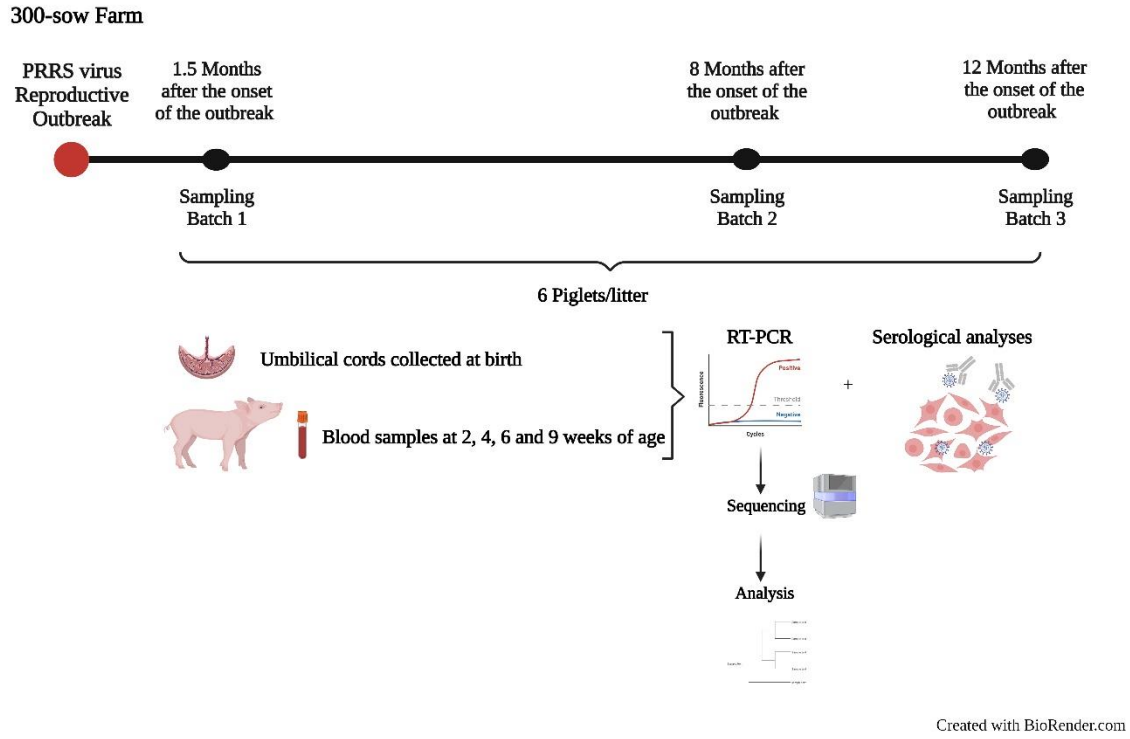

Figure 1. Timeline and design of the present study. The figure shows a summary of the follow-up performed in the farm after the onset of a PRRSV-1 reproductive outbreak. Three batches of piglets were followed 1.5 (Batch 1), 8 (Batch 2), and 12 months after the outbreak (Batch 3) from birth to 9 weeks of age. Samples were used to detect PRRSV by RT-qPCR, sequence, and assess the animals' serological status against PRRSV.

A follow-up of the disease and the viral circulation started 6 weeks after the onset of the outbreak. The first sampling was conducted one and a half months after the onset of the outbreak (Batch 1, 72 piglets from 11 litters), the second one 8 months after the onset of the outbreak (Batch 2, 74 piglets from 9 litters), and the third one 1 year after the onset of the outbreak (Batch 3, 70 animals from 10 litters). At least 6 piglets were selected based on the probability sampling principle within each litter. In each batch, sows of different parity were included (from parity one to eleven overall). The sampling consisted of the collection of umbilical cords (UC) at birth and blood samples at 2, 4, 6, and 9 weeks of age. All animals included in the follow-up were ear tagged to identify them individually.

3.3.2. Detection of porcine reproductive and respiratory syndrome virus by RT-qPCR and viral isolation.

Umbilical cords (approximately 4 cm) were collected soon after birth and submerged in a tube containing sterile PBS with antibiotics. Scissors and forceps used for sample collection were decontaminated after each use by submerging them in 2% sodium hypochlorite and were then rinsed with distilled water. The collected UC were minced with sterile scissors and blades, and then the suspension was vortexed and centrifuged at 12,000 × g. The supernatants were aliquoted and stored at −80°C until used. Blood was collected by cava or jugular vein puncture depending on the age of the animal. Serum was separated by centrifugation after clotting and aliquoted to store at −80°C.

Viral RNA was extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel, Germany) according to the manufacturer's directions, with an elution volume of 50 µL. The presence of PRRSV RNA in the sample was assessed by a commercial RT-qPCR kit aimed to detect both PRRSV-1 and PRRSV-2 (LSI VetMAX™ PRRSV EU/NA kit, Thermo Fisher Scientific, United States) according to the instructions provided. An internal positive control (IPC) was included in each reaction, in addition to positive and negative controls for the RNA extraction and the PCR (samples with a known viral amount and DEPC-water). Samples with C_t values ≤37 were considered positive, and between 37.1 and 39.9 were considered inconclusive. With this data, the incidence for each observation period was calculated as the proportion of infected animals over the number of susceptible animals in each time period.

Virus was isolated in porcine alveolar macrophages (PAM) for the subsequent whole genome sequencing. PAM were isolated by bronchoalveolar lavage as reported previously (Mayer and Lam, 1984). To avoid biasing the results, only a single passage was carried out. According to the previous report (Cortey et al., 2018), discrepancies between sample-to-sequence and PAM isolate-to-sequence were 1–3 per 10−4 nucleotides with this method. For UC, all positive samples with C_t values ≤32.0 were selected for further isolation in PAM. For sera, isolation was attempted from all animals infected at the farrowing units and from their pen-mates that were RT-qPCR positive during the study. Cell culture supernatants of the successfully isolated samples were collected, aliquoted, and stored at −80°C.

3.3.3. Sequencing.

For Batch 1 and 2, ORF5, that encodes for the major envelope protein of the virus and has been traditionally used for phylogenetic analyses of PRRSV, was amplified and Sanger sequenced for all positive samples with a C_t value ≤32.0 using a previously reported protocol (Mateu et al., 2003). For Batch 3, at least 50% of the samples with the required Ct were sequenced for ORF5 (randomly selected). For Batch 1, viral non-structural protein 2 (nsp2) and nsp9 segments, a highly variable and a highly conserved segment of the genome, respectively, were also partially sequenced using tailor-made oligonucleotide primers (Supplementary Table S1).

Whole-genome-based analyses may provide a fuller picture of PRRSV genetic diversity and evolution. Therefore, viral RNA was extracted from the isolates using the TRIzol™ reagent (Thermo Fisher Scientific, United States) following the manufacturer's instructions, with an elution volume of 20 μL. The viral RNA was used for next generation sequencing (NGS) using Illumina Miseq without performing any previous amplification. Briefly, the protocol developed in five steps. First, the genomic library was constructed using a commercial protocol and reagents (Protocol for use with Purified mRNA or rRNA Depleted RNA and NEBNext® Ultra™ II RNA Library Prep Kit for Illumina®, New England Biolabs, United States). After the NGS run, sequences of low quality were trimmed (QC < 20) using Trimmomatic© (Bolger et al., 2014; RRID:SCR_011848). Then, reads were mapped against a reference sequence (Burrows-Wheeler Aligner applying the BWA-MEM algorithm for long reads; Li and Durbin, 2010; RRID:SCR_010910). The reference sequence was produced from the earliest available isolate obtained during the outbreak by de novo assembly using SPAdes© (Bankevich et al., 2012; RRID:SCR_000131). In the fourth step, variant calling was performed with SnpSift© (Cingolani et al., 2012; RRID:SCR_015624) to determine the frequency of each nucleotide at each position of the reference genome. Finally, the viral quasi-species was constructed in fasta format, and the consensus sequence was obtained using Consensus© software, available at www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html. The consensus sequences for the whole genomes and the ORF5, the nsp2, and the nsp9 sequences were submitted to GenBank with the Accession Numbers OP688189 - OP688223, OP688224 - OP688357, OP822784 - OP822837, and OP822838 - OP822859, respectively. The NGS raw sequence reads were also submitted to GenBank with the BioProject ID PRJNA915491.

3.3.4. Sequence analyses.

For comparative purposes, a selection of available complete genome and ORF5 sequences of PRRSV-1 strains including the five PRRSV vaccines commercially licensed in Spain were downloaded from Genbank (Supplementary Table S2). Phylogenetic trees were constructed using MrBayes (Bayesian inference with 1,000,000 iterations; Ronquist et al., 2012; RRID:SCR_012067) and p-distances were calculated using MEGA X (Tamura et al., 2021; RRID:SCR_000667).

For a detailed phylogenetic analysis of the sequences obtained in this study, phylogenetic trees were built up for the whole genome sequence and each viral nsp and ORF using MEGA X (maximum likelihood method, general time reversible model with 1,000 iterations and pairwise deletion) and MrBayes (Bayesian inference with 1,000,000 iterations). P-distances were calculated with MEGA X to determine the range of inter-and intra-batch and clade diversity for the whole genome sequences and the viral segments. The substitution rate was estimated for the whole genome and each segment using BEAST (three independent Markov chain Monte Carlo Bayesian simulations per segment, 108 steps each; Suchard et al., 2018; RRID:SCR_010228). The existence of potential recombination in the whole genome sequences was analyzed using GARD (Pond et al., 2006) and RDP5.0 (Martin et al., 2020). In addition, a comparison of the amino acid composition of the predicted protein sequences was performed between batches, clades, and the vaccine used in the farm.

3.3.5. Transmission chains.

Since animals were ear-tagged and all movements of animals between pens were recorded, the chain of infection was tracked through the phylogenetic sequences, pens where animals were located, and the possible direct contacts animals had before the infection. Traceable horizontal transmission was considered to occur when the variant in a newly emerged case was the same as the one presented in a direct-contact pig at the previous observation timepoint. When more than one animal could be considered the source of infection (for example two pigs with the same viral variant had direct contacts), the new case was considered traceable, but the source animal was non-identifiable. With

this information, maps of transmission were built, and the incidence of each variant could be estimated approximately.

3.3.6. Intrahost diversity.

The viral quasi-species inferred from the NGS runs were used to analyze the distribution and evolution of the intrahost diversity. Among the individuals sampled, two consecutive quasi-species were available for five of them. For every viral quasi-species, the nucleotide frequencies per position obtained in the fourth step of the NGS procedure described in section 2.3 were summarized. The analyses focused on positions with high diversity, arbitrarily defined as those where the frequencies of the mutations present were larger than 10%. Finally, the positions identified were located within the genome.

3.3.7. Viral replication kinetics.

To assess the replication kinetics of the different viral variants found in the farm, one isolate was used of each clade. PAM were inoculated at multiplicity of infection (MOI) of 0.01 or medium for negative controls. Three replicates per clade and negative controls were included for each harvest time-point. Supernatants were harvested at 24, 48, 72, and 96 h post-infection. Viral RNA was extracted from the supernatants, and then quantified by RT-qPCR as described in 2.2. An internal positive control was included in all extractions. C_t values were plotted against the results of a series of dilutions of the same virus.

3.3.8. Serological analyses.

All serum samples in the study were analyzed by ELISA (IDEXX PRRS X3 Ab Test, IDEXX, United States) to determine the animals' serological status against PRRSV at the moment of infection and the seroconversion if infected. The ELISA results were expressed as S/P ratio values, namely, the ratio between the corrected optical density (OD) of a given sample (OD sample-OD negative control) over the corrected OD of the positive control included in the kit (OD positive control-OD negative control). This S/P ratio allowed semi-quantification and comparison of the results obtained with the different sera. An S/P ratio ≥ 0.40 was considered positive for PRRSV antibody. Additionally, a selection

of sera of animals of Batch 1 and 3 was used to assess the capability for neutralizing the predominant viral variant at the end of the study. This selection considered: (i) samples from born-infected animals having viremias longer than 5 weeks, (ii) samples from infected animals at the farrowing units (before weaning) and their PRRSV-negative siblings, (iii) samples from PRRSV-negative animals at 2 weeks of age, (iv) samples taken at 4 and 9 weeks of age of animals infected at 4 weeks of age, or (v) samples from the offspring of sows that were present in the sampling of both Batch 1 and 3 as representative of animals that potentially could have been in contact with the studied viral variants.

Viral neutralization test (VNT) was performed using the protocol described by Yoon et al. (1994) with minor modifications. The virus used for this test was the predominant variant in Batch 3 that had been previously adapted to replication in MARC-145 cells and was fully sequenced following the protocol abovementioned. Only NAb titers ≥ 2 $\log_2$ were considered to be relevant. Blood samples of sows ($n = 96$), which were collected after the present study (2–3 months after Batch 3 sampling) for routine health monitoring purposes, were also examined in the VNT.

3.3.9. Statistical analyses.

The number of PRRSV vertical transmissions and mortality was compared between batches using a Chi-square test with Yates correction. S/P ratio values at 2 and 9 weeks of age and at 2 weeks of age based on the parity of the sow were compared between batches. Neutralization titers were $\log_2$ normalized and were compared. All statistical analyses were carried out using GraphPad Prism v9 (RRID:SCR_002798).

3.4. RESULTS

3.4.1. Viral circulation in the farm.

Incidence of PRRSV in the different batches is shown in Figure 2. In Batch 1, 11 litters including 72 animals were examined. PRRSV-positive animals were detected in 4/11 litters (36.4%; CI95%: 12.4–68.4%) at birth ($n = 11$; between 1 and 5 positive animals per litter; average C_t value 30.8 ± 4.0). In 1/11 litter, inconclusive RT-qPCR results ($Ct > 37.0$) were obtained from the UC of the new-born piglets, but infection was confirmed at

2 weeks of age. During the observation period of this batch, 58 animals (80.6%; CI95%: 69.2–88.6%) were infected, and 24 (33.3%; CI95%: 22.9–45.5%) died in the period between birth and 9 weeks of age, 10 of which in the first 2 weeks of age (41.7% of the total mortality). In Batch 2, viral circulation was very low with only 8/74 animals testing positive overall (10.8%; CI95%: 5.1–20.7%, $p < 0.05$) corresponding to two UC and one, two, and three animals at 4, 6, and 9 weeks of age, respectively, with C_t values >31.5. Mortality for this batch was 21.6% (CI95%: 13.2–33.0%). In Batch 3 ($n = 70$), positive piglets were found in 6 litters at birth (60.0%; CI95%: 27.4–86.3%; average Ct 35.6 ± 1.0) and results of UC were inconclusive in two other litters. During the observation period, 55 animals (78.6%; CI95%: 66.8–87.1%) were infected. Mortality for this batch was 18.6% ($p < 0.05$).

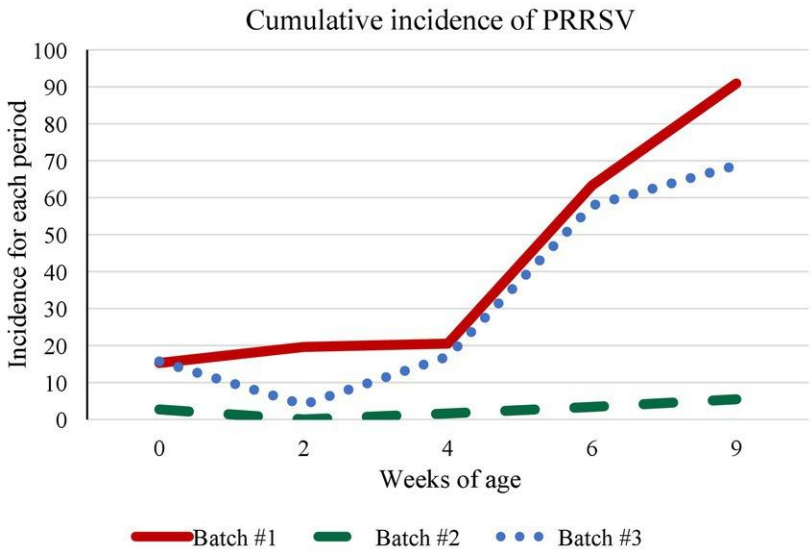

Figure 2. Evolution of the cumulative incidence of PRRSV-1. The graph shows the cumulative incidence of PRRSV-1 for each observation period (2, 4, 6, and 9 weeks of age) per batch. The incidence was calculated as the proportion of infected animals over the number of susceptible animals in a given time period.

3.4.2. Phylogenetic and evolutionary analyses.

The phylogenetic analyses of the sequences obtained in this study together with the PRRSV-1 reference sequences and the five vaccines commercially licensed in Spain indicated that the circulating virus strain in the farm of the present study did not derive from the vaccine used in the farm (Supplementary Figures S1, S2) and had a whole genome similarity of 86.5% to the vaccine strain (Supplementary Table S3). In addition, the analysis showed that variants of the same virus strain were circulating throughout the follow-up and there was no novel introduction of a different virus.

A Bayesian phylogenetic tree inferred from the ORF5 sequences obtained from born-infected animals and the new infection cases is shown in Figure 3. No quality sequences could be obtained from Batch 2 samples. The phylogenetic analysis of ORF5 showed a clear differentiation of Batch 1 sequences from Batch 3 sequences. In Batch 1, four significantly different clades were distinguished and 3 of them included variants detected in born-infected animals (animal 515 in Clade 1, animals 526 and 527 in Clade 2, and animals 518–522 in Clade 3). Similar results were seen for nsp2 and nsp9 segments (Supplementary Figures S3, S4). The analysis of the whole genome sequences confirmed that Batch 1 and Batch 3 sequences formed two distinct branches (Supplementary Figure S5), and the four abovementioned clades could also be identified in Batch 1. The phylogenetic trees based on maximum likelihood method from ORF5, nsp2, nsp9, and whole genome sequences are shown in Supplementary Figures S6–S9, respectively.

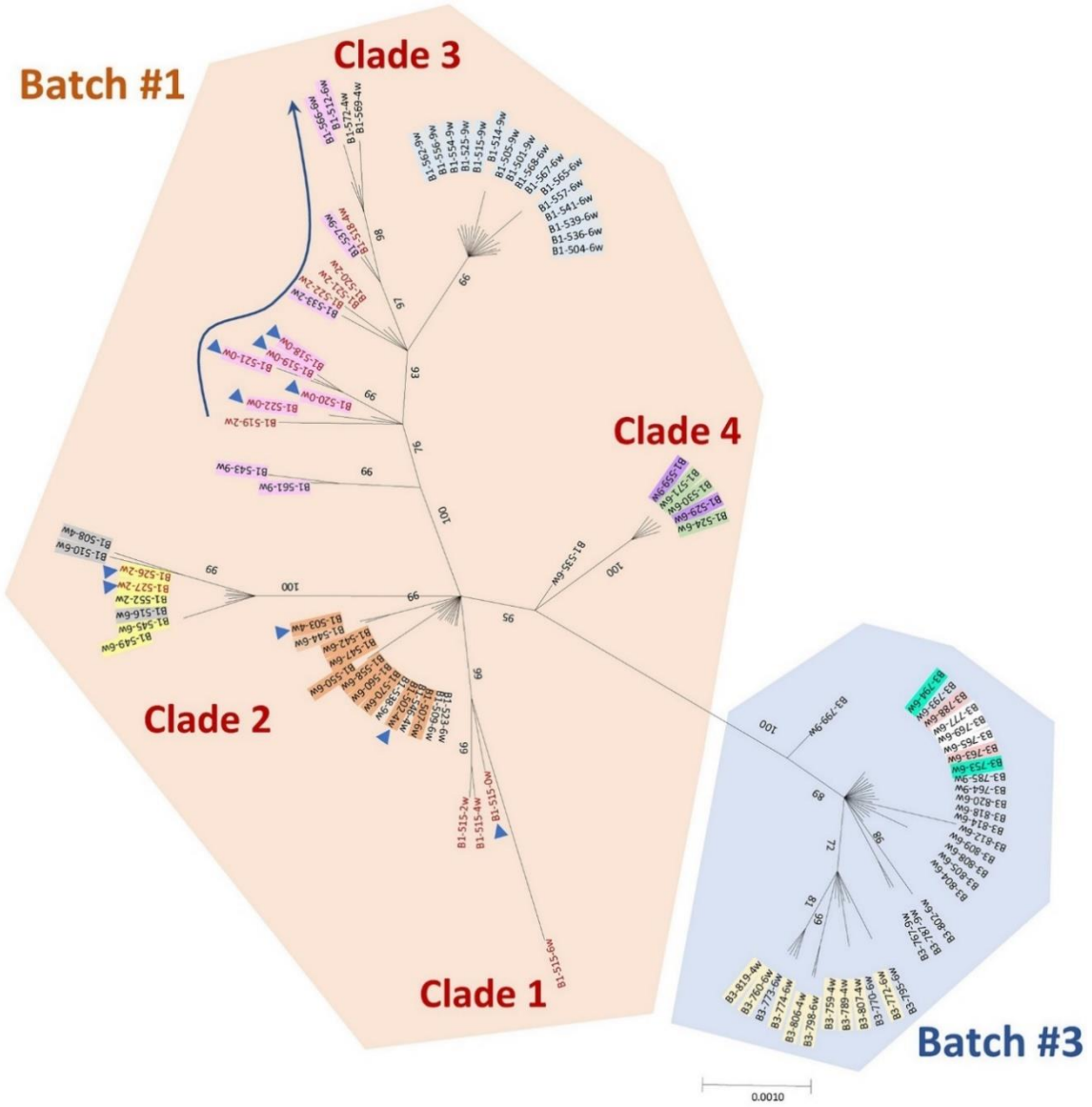

Figure 3. Bayesian phylogenetic tree based on the nucleotide sequences of PRRSV-1 ORF5 (gene size of 606 bp) using MrBayes (1,000,000 iterations). ORF5 sequences obtained from born-infected animals with long viremias (in red) and the new infection cases (in black) are included. The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. Sequences from vertical transmission events are marked with a blue triangle. The shaded sequences with the same color correspond to animals belonging to a transmission chain. Only posterior probability values >70% are shown.

Using ORF5, the net distance between Batch 1 and 3 was 0.0135 ± 0.0041 (Table 1). The estimated annual drift for ORF5 was 1.108% (Table 2). The calculation of the nucleotide mean distance within Batch 1 (p-distance 0.0078 ± 0.0020) was 3 times higher than in Batch 3 (p-distance 0.0023 ± 0.0009). When the whole genome was used for the calculations of the nucleotide mean p-distance within each batch, similar values were obtained (0.0058 ± 0.0004 and 0.0020 ± 0.0002, for Batch 2 and 3 respectively). The estimated annual drift for the whole genome was 0.638%. Furthermore, no recombinant events among the sequences were detected in the analysis.

Table 1. Similarities (p-distance ± standard error) within and between Batch 1 and 3. The table shows the nucleotide mean differences within and between the examined batches of PRRSV-1 whole genome consensus sequences and each segment of the viral genome. Nsp, non-structural protein.

Segment	Batch1	Batch3	Batch 1 *vs* 3
Genome	0.0058±0.0004	0.002±0.0002	0.0131±0.0011
ORF1a			
nsp1a	0.0057±0.002	0.0037±0.0015	0.0155±0.0049
nsp1b	0.0071±0.0018	0.0024±0.0011	0.0127±0.0041
nsp2	0.006±0.0009	0.0025±0.0005	0.0189±0.0025
nsp2TF	0.0068±0.0009	0.003±0.0006	0.0189±0.0022
nsp3	0.0045±0.0015	0.0008±0.0005	0.0104±0.0031
nsp4	0.0088±0.0024	0.0009±0.0005	0.0158±0.0046
nsp5	0.0068±0.0022	0.0021±0.001	0.0141±0.0047
nsp6	0.0102±0.0103	0±0	0.0027±0.0027
nsp7a	0.0092±0.0028	0.0012±0.0007	0.0116±0.0046
nsp7b	0.0033±0.0018	0.001±0.0007	0.0089±0.0046
nsp8	0.0045±0.0024	0.0032±0.0031	0.0039±0.0036
ORF1b			
nsp9	0.0042±0.0009	0.0015±0.0005	0.0124±0.0024
nsp10	0.0043±0.0009	0.0015±0.0006	0.0087±0.0023
nsp11	0.0019±0.0008	0.0021±0.0007	0.0118±0.0041
nsp12	0.0077±0.0022	0.003±0.0012	0.0232±0.0064
ORF2a	0.0048±0.0014	0.0012±0.0005	0.0053±0.0022
ORF2b	0.0033±0.0024	0.0009±0.0008	0.0052±0.0044
ORF3	0.0085±0.0021	0.0049±0.0012	0.0076±0.0025
ORF4	0.0068±0.0019	0.0017±0.0007	0.0094±0.0037
ORF5a	0.0097±0.0056	0.0014±0.0013	0.028±0.0137
ORF5	0.0078±0.002	0.0023±0.0009	0.0135±0.0041
ORF6	0.0091±0.0025	0.0017±0.0009	0.0174±0.0052
ORF7	0.0053±0.0023	0.0009±0.0008	0.0065±0.0036
3UTR	0.0283±0.0078	0.0151±0.0067	0.0023±0.0018
5UTR	0.0056±0.0025	0±0	0.0185±0.0088

Table 2. Yearly drift and 95% confidence interval per segment of the viral genome. Calculations based on substitution rate estimations obtained by BEAST (Suchard et al., 2018; RRID:SCR_010228), after three independent MCMC Bayesian simulations per segment, 10^8 steps each, considering the default prior parameters provided by the software.

Segment	95%-Low	%	95%-High
Genome	0.434	0.638	0.867
ORF1a			
nsp1a	0.265	0.465	1.029
nsp1b	0.084	0.283	0.627
nsp2	0.229	0.484	0.826
nsp2TF	0.045	0.109	0.197
nsp3	0.311	0.707	1.231
nsp4	0.457	0.966	1.674
nsp5	0.276	1.115	2.352
nsp6	0.000	0.006	1.105
nsp7a	0.122	0.562	1.369
nsp7b	0.183	0.694	1.523
nsp8	0.056	0.501	1.393
ORF1b			
nsp9	0.001	0.361	0.863
nsp10	0.351	0.714	1.166
nsp11	0.693	1.391	2.299
nsp12	0.339	1.452	3.091
ORF2a	0.272	0.671	1.218
ORF2b	0.059	0.475	1.284
ORF3	0.265	0.527	0.872
ORF4	0.309	0.937	1.748
ORF5a	0.983	2.759	5.281
ORF5	0.712	1.108	1.568
ORF6	0.266	0.887	1.728
ORF7	0.164	0.517	1.041

Amino acid composition differences of the viral proteins between clades of Batch 1 and Batch 3 are shown in Supplementary Table S4. All sequences presented a deletion of 5 amino acids in nsp2 between position 347–351 referred to the prototype PRRSV-1 strain Lelystad (LV; NC043487). There were in total 73 amino acid differences between clades of Batch 1, and 61 differences between Batch 1 and 3. Two amino acid variations in GP4 (amino acid positions 65 and 69) and two in GP5 (amino acid position 41 and 46) affected known neutralizing epitopes (Meulenberg et al., 1997; Plagemann, 2004; Vanhee et al., 2011). Additionally, one of these variations in GP5 (amino acid position 46) was located at a known glycosylation site (Meulenberg et al., 1995; Wissink et al., 2003, 2004). When comparing the predicted amino acid composition of the viral proteins between the virus

circulating on the farm and the used vaccine strain (Supplementary Table S5), there were in total 589 amino acid differences. Thirteen amino acid changes in GP4 and 2 in GP5 affected known neutralizing epitopes.

3.4.3. Intrahost diversity.

Intrahost diversity was examined at two different timepoints in 5 animals that tested positive for the virus at least twice (2 in Batch 1 and 3 in Batch 3). The results showed that the number of highly variable positions was higher in the quasi-species inferred from the sequences of first batch animals (64) compared to the third batch animals (22.5) (Table 3). Also, the positions were not evenly distributed throughout the genome and the segment corresponding to nsp1a (540 nucleotides) accumulated most of those highly variable positions, 55% for Batch 1 animals and one third for Batch 3 animals. Of note, no significant differences were observed between the first and the second timepoint regarding those highly variable positions in each animal.

Table 3. Summary of highly variable positions (positions showing overall mutation frequencies higher than 10%) in the 10 viral quasi-species analyzed, and location of those positions in the PRRSV-1 genome. The table shows the results for two animals of Batch 1 and three animals of Batch 3 that tested positive in two timepoints.

Batch	Batch 1				Batch 3					
Animal	518		521		769		770		798	
Age at sampling	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks	9 weeks	6 weeks	9 weeks	6 weeks	9 weeks
ORF1a										
nsp1a	29	33	38	27	8	2	7	2	3	3
nsp1b	1	0	0	2	0	0	0	3	1	0
nsp2	17	2	6	9	2	0	1	17	4	7
nsp3	3	0	3	0	0	0	0	3	0	0
nsp4	2	0	1	1	1	0	0	2	0	2
nsp5	4	0	0	2	0	0	0	3	3	0
nsp6	0	0	0	0	0	0	0	0	0	0
nsp7a	6	1	1	0	1	0	0	0	0	1
nsp7b	1	0	0	1	0	0	0	0	1	0
nsp8	1	0	0	0	0	0	0	2	0	1
ORF1b										
nsp9	5	1	3	6	0	0	2	4	0	2
nsp10	3	0	1	3	0	0	0	3	0	0
nsp11	0	0	0	0	1	0	1	0	1	0
nsp12	2	0	5	0	0	0	0	0	1	0
ORF2a	1	1	1	1	1	1	0	9	8	1
ORF2b	0	0	0	0	0	0	0	0	0	0
ORF3	1	1	0	1	2	0	1	3	2	1
ORF4	0	0	1	0	0	0	0	2	0	1
ORF5	5	0	3	4	0	0	0	0	1	2
ORF5a	3	0	2	1	0	0	0	0	0	1
ORF6	6	0	0	2	1	0	0	0	0	0
ORF7	0	0	0	2	0	0	0	0	2	1
Number of highly variable positions	90	39	65	62	17	3	12	53	27	23

3.4.4. Transmission chains.

The phylogenetic analyses together with the location records of animals at each timepoint led to the identification of possible direct contacts of the infected individuals (shown in Supplementary Material Animation). For Batch 1, the transmission chain was possible to be traced back in 51 out of the 58 infection cases (87.9%), while for Batch 3, 16 out of the 34 (47.1%) sequenced cases. In Batch 1, nine animals were identified as the source of 24 out of 28 horizontal transmission events, and 4/9 of these animals derived from vertical transmission events. In Batch 3, all identified transmissions could be traced to 8 animals. In both batches, most of the identified virus donors transmitted the infection to 1–3 animals; however, in Batch 1, one animal (number 502) was identified as the source of infection for 8 cases, and in Batch 3, one animal (number 777) was identified as the source of infection for 4 cases.

It is worth noting that in Batch 1, 4 animals that were born viremic remained so until the end of the observation period. Of these, three contributed to infect pen-mates but the animal number 515 was not identified as the source of any transmission. Moreover, this animal harbored a viral variant from birth to 6 weeks of age and a different one at 9 weeks of age, suggesting a new infection with a variant horizontally transmitted by another animal.

3.4.5. Viral replication kinetics.

To evaluate if differences in transmission were related to the replication capability of the viral variants, replication kinetics were examined for one isolate of each clade (Figure 4). The different clades displayed generally a similar replicative fitness in cell culture. The isolate of Clade 2 might have shown a higher early infectivity, which could suggest a higher affinity to the viral receptors, although it did not result in a higher predominance among the population.

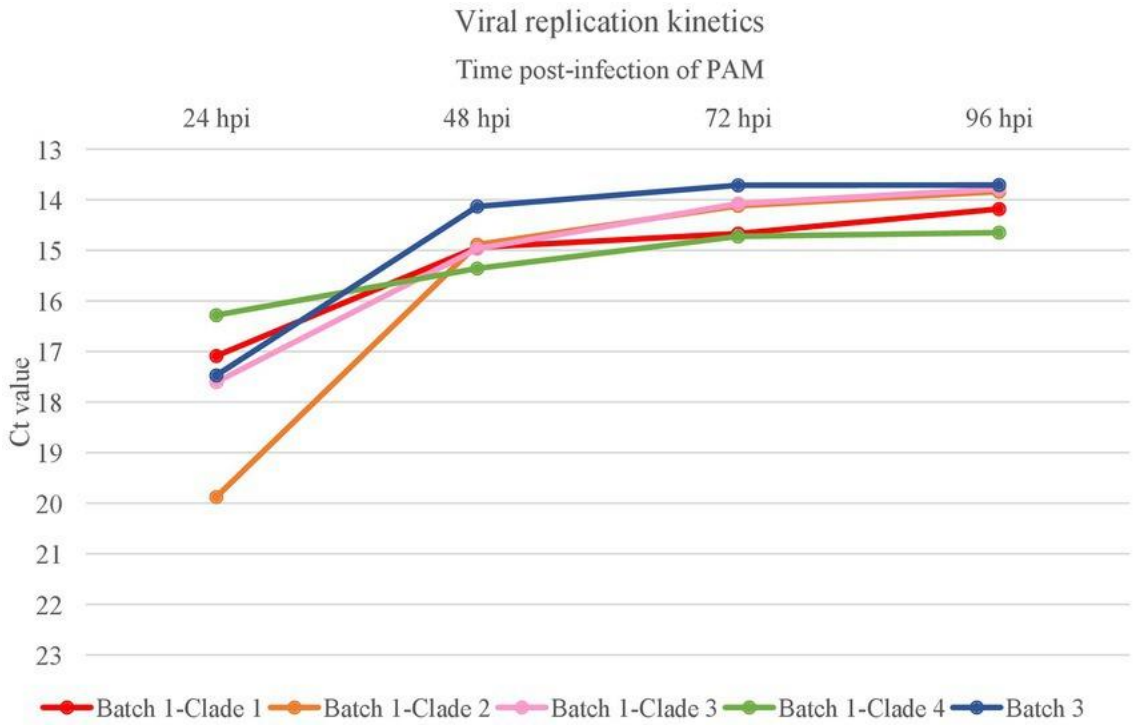

Figure 4. Virus replication kinetics of the different viral clades identified in the farm. The graph shows the kinetic replication curves of variants (n = 4) from each clade of Batch 1 and the variant (n = 1) in Batch 3 based on C_t values of the supernatants harvested at 24-, 48-, 72-, and 96-h post-infection (hpi) of porcine alveolar macrophages (PAM) at multiplicity of infection of 0.01.

3.4.6. Serological analyses.

3.4.6.1. ELISA antibodies.

When the presence of anti-PRRSV antibodies was assessed by ELISA using the sera collected from piglets at 2 and 9 weeks of age, significant differences in the S/P ratio values were noticed. Thus, for 2-week-old piglets, the highest S/P ratios were found in animals from Batch 1, followed by the animals of Batch 3, and the animals of Batch 2 (Figure 5). At 9 weeks of age, S/P ratios were similar between animals of Batch 1 and 3, but almost all pigs in Batch 2 were seronegative, indicating that PRRSV circulation was limited in the nurseries at that time. When the 2-week-old piglets in Batch 2 were examined based on the parity of the sow, the statistical analysis indicated that the offspring of sows ≤6 parity had significantly lower antibody levels (Supplementary Figure S4).

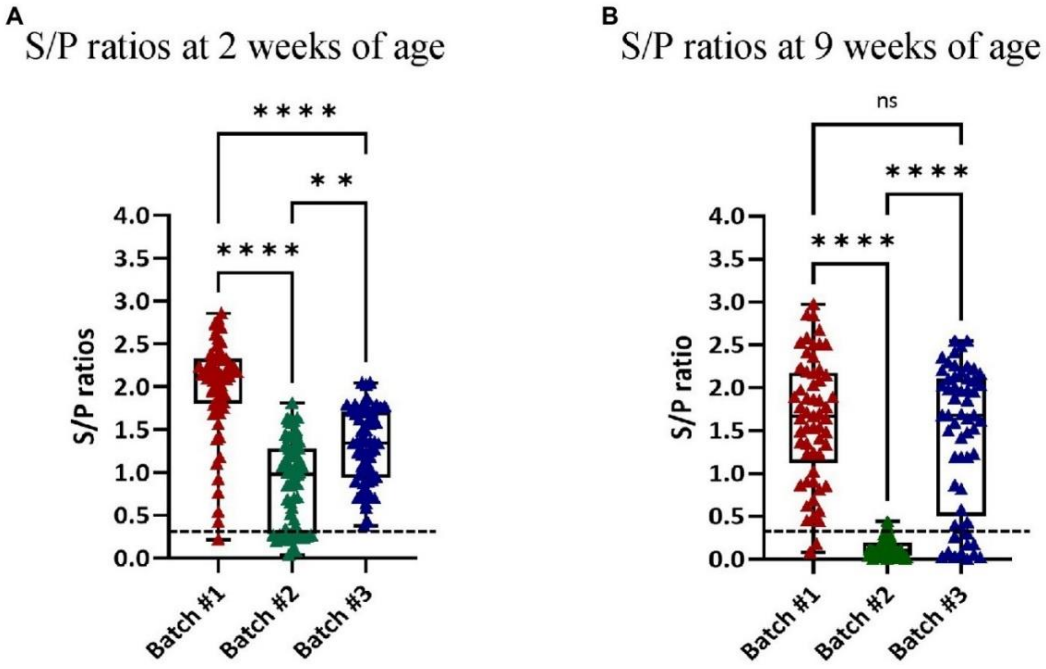

Figure 5. S/P ratios of the piglets as determined by ELISA. Each dot represents an examined individual. S/P ratio values ≥0.4 are considered positive (dotted line). The graphs show the S/P ratio values of the piglets at 2 (A) and 9 (B) weeks of age in each of the examined batches. ns = not significant.

3.4.6.2. Neutralizing antibodies.

The levels of NAb were examined in different groups of animals. Regarding animals that were born-infected (n = 7), most of them remained negative all over the observation period and only one was detected as positive at week 6 of age (2 $\log_2$). The second examined group were those animals that were infected in the farrowing units (n = 16) compared to their siblings (n = 21). Most of the animals in both groups were negative, indicating NAb were not related to their infection status. Similarly, the examined uninfected animals at 2 weeks of age did not have relevant NAb titers. In addition, average titers at 2 weeks of age were not significantly different when comparing Batch 1 and 3. Next, we examined if animals infected at 4 weeks of age had developed NAb 5 weeks later (n = 17). Of the 17 examined animals, only 3 (17.6%) had developed NAb against the circulating strain of PRRSV (one in Batch 1 and two in Batch 3). Figure 5 summarizes the results of the VNT analyses.

In addition, we assessed NAb in the offspring (2 weeks of age) of six sows sampled in both Batch 1 and Batch 3 to ascertain whether maternal-to-fetal transfer of NAb increased with time after potential contact with different viral variants circulating in the

farm. Of those six sows, one gave birth to infected piglets in Batch 1, a second gave birth to infected piglets in Batch 3, and a third gave birth to infected piglets in Batch 1 and 3 (Table 4). When the offspring of the sows were examined in Batch 1, positive VNT titers were found in four out of six litters (11/21 animals within the positive litters, 52.4%) with titers ranging from 2 log_2 to 5 log_2. When the offspring of the same sows were examined in Batch 3, only 2 litters had positive animals (8/12, 66.7% within the positive litters) with titers ranging from 2 log_2 to 5 log_2. It is worth noting that the offspring of 2 of the 3 sows that delivered infected piglets at birth tested negative in the VNT in both Batch 1 and 3. Moreover, the offspring of one sow of parities 9 to 11 (Batch 1 and Batch 3, respectively) always tested negative.

Table 4. Parity, PRRSV-1 vertical transmission, and neutralizing antibodies titers of the offspring (2 weeks of age) of six sows that were present in both Batch 1 and Batch 3. Titers are shown as log_2 values. Neg., negative.

Sow number	Parities	Vertical transmission	Nº piglets examined	VNT titers	
	Batch 1 vs. Batch 3	Yes/No, batch	Batch 1 vs. Batch 3	Batch 1	Batch 3
512	9 - 11	Yes, Batch 3	3 – 5	Neg.	Neg.
765	4 - 6	Yes, Batch 1 & 3	4 – 6	Neg.	Neg.
783	4 - 6	Yes, Batch 1	4 – 5	Neg. to 5 log_2	Neg.
552	9 – 11	No	4 – 6	Neg. to 3 log_2	Neg. to 2 log_2
635	7 – 9	No	3 – 6	2 -3 log_2	Neg. to 5 log_2
893	1 – 3	No	5 – 4	Neg. to 4 log_2	Neg.

Finally, we examined the presence of NAb in sera collected from sows for health monitoring purposes over one year after the original outbreak. At that moment, most of the sows had low VNT titers or were negative (Figure 6) but no differences could be determined regarding parity.

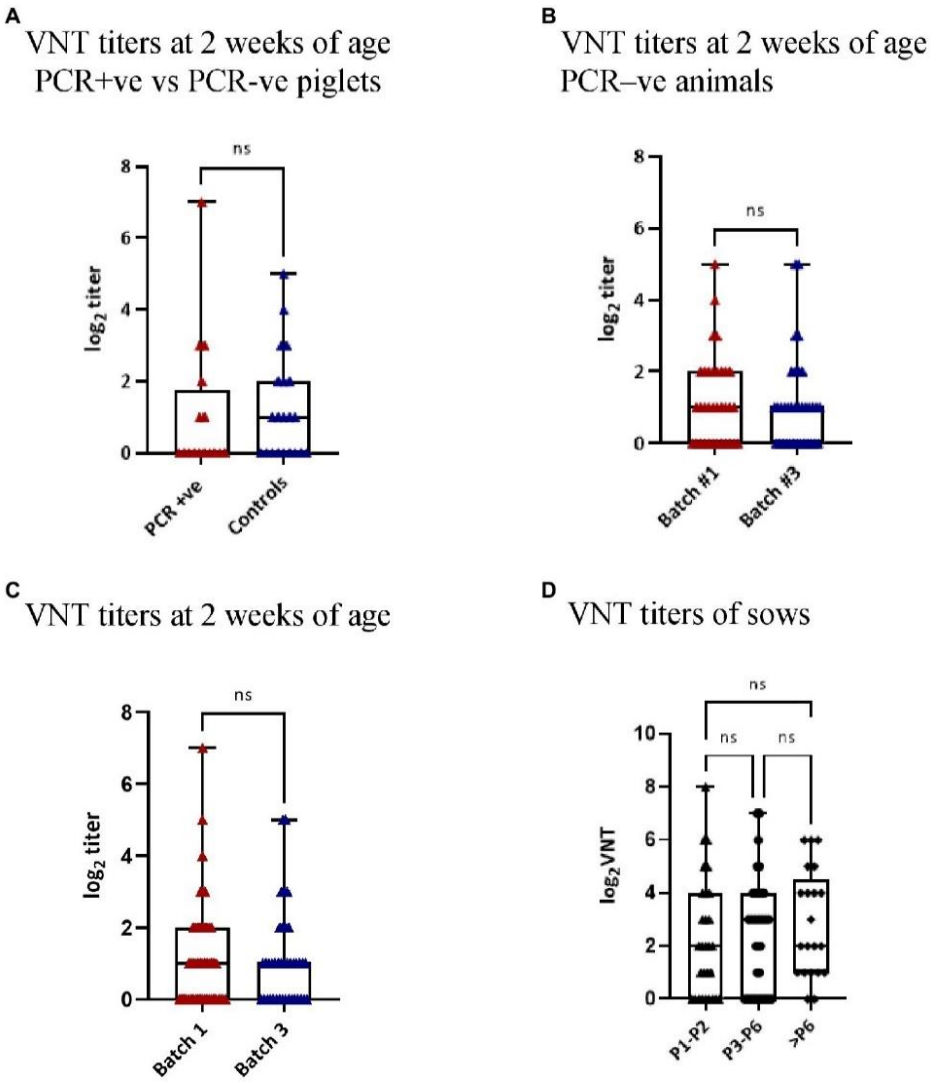

Figure 6. Virus neutralization titers in the different groups. Each dot represents an examined individual. Titers are shown as log_2 values and only NAb titers ≥2 log_2 were considered to be relevant. (A) The graph compares the VNT titers of the PCR positive (PCR + ve) animals at 2 weeks of age and their PCR negative (PCR-ve) siblings. (B) The graph shows the VNT titers of the PCR negative (PCR-ve) animals at 2 weeks of age in Batch 1 and 3. (C) The graph shows the VNT titers of 2-week-old piglets of Batch 1 and 3. (D) The graph shows the VNT titers according to the parity of the 96 sows sampled one year after the onset of the outbreak. ns = not significant.

3.5. DISCUSSION

The introduction of PRRSV in a naïve farm usually results in a major reproductive outbreak. After a variable period, the reproductive performance improves, and the farm enters in an endemic state in which the disease is mostly seen in nurseries. It is commonly assumed that the vertical transmission is the key element for the maintenance of the infection in the farm since it results in the birth of infected animals. These piglets can be viremic for a long period and are thought to be efficient transmitters of the disease to their

pen-mates. Most of the control strategies for the successful elimination of PRRSV from the farm rely on depopulation or on strategies like the load, immunization, and closure of the farm. In other words, it is assumed that after immunizing all the animals present in the farm and closing the farm to the entrance of new susceptible animals, the circulation of the infection will cease. The duration of the closing period is a key element in those strategies. However, in many farms, depopulation or closure strategies are not economically feasible and other approaches are needed.

In those other cases, monitoring, vaccination, and biosecurity measures are essential for the control. Monitoring usually focuses on the detection of infected animals and the hotspots of viral circulation (replacement gilts, sows, and nurseries). Additionally, it uses sequencing as a tool to determine if the viral circulation in the farm is caused by the previously resident strain or by a newly introduced one. Sometimes the same strain reemerges after a period in which viral circulation seemed to be contained. Our knowledge of the microepidemiology of PRRSV within the farm over time is still limited.

The present study focused on the examination of the diversification of the virus after an outbreak in a vaccinated farm, relating the viral evolution with the variations in the incidence of the infection and the development of an immune response to the virus. For that purpose, we followed a farm for one year after a reproductive outbreak.

We started the study approximately 6 weeks after the initial detection of the outbreak. At that moment about one third of the examined litters had infected animals at birth, indicating a high proportion of susceptible sows. The phylogenetic analysis at that point showed a high viral diversity with 4 recognizable different clades of the virus. Three of these clades were identified in new-borns, suggesting the existence of a founder effect. These founder effects were described initially in HIV and hepatitis C virus and can represent population or selection bottlenecks (Weiner et al., 1993; Gerotto et al., 2006; Russell et al., 2011; Naidoo et al., 2017). In other words, the transmission of the virus from one individual to the other through mucosal surfaces or through the placenta may result in a quantitative selection of viral particles (only few can pass through) or in a qualitative selection (only some viral particles with specific characteristics can be transmitted through the mucosal surfaces). In our case, we cannot know the nature of these bottlenecks but the fact that the sows had been multiple vaccinated and might have been infected previously suggest that both types of bottlenecks could have acted. In a previous work (Cortey et al.,

2018) we have shown that selection bottlenecks for PRRSV can exist in vaccinated animals.

After the initial outbreak and the high spread of the infection in the farrowing units and nurseries in the first batch, in Batch 2 (around 6 months later), the viral circulation was limited. The few animals in this batch that tested positive in the RT-qPCR produced very high C_t values, indicating low viral loads or even detection of environmental virus for example in the case of UC collection. The key question is why the viral circulation almost stopped. The most intuitive answer would be that the circulation ceased because the population was immune. However, when the animals of that batch were serologically examined, the antibody levels were low in 2-week-old piglets, suggesting that the sows did not have a strong humoral immunity against the virus. Whether this was because of a rapid decline of herd immunity after the outbreak or because of the poor induction of antibodies by that particular virus isolate, or both, is difficult to say. In any case, the absence of antibodies against PRRSV in most of the 9-week-old animals of Batch 2 is a strong indication that the virus was not circulating indeed. Moreover, as we will discuss later, the fact that both piglets and sows had low levels of NAb against the circulating virus suggests that humoral immunity might not be a complete indicator for understanding how immunity affects viral persistence in the herd. Although correlates of protection against PRRSV have not been fully elucidated, Lopez et al. (2007) showed that passive transfer of neutralizing antibodies results in protection in a homologous challenge model when titers of 1:8–1:16 are reached. However, there is evidence that cell mediated immunity also plays a role in protection against PRRSV-1 infection in the homologous and heterologous models (Díaz et al., 2012).

Then, we examined Batch 3 four months after Batch 2. Surprisingly, the infection not only did not disappear but re-emerged, and at the end of the nursery period most animals had been infected. How did this happen? The phylogenetic analysis of Batch 3 isolates clearly showed that a new variant of the resident virus gained predominance. However, the variant found at this moment was much less diverse than any variant found in Batch 1, indicating a strong selection process that happened very close to the timepoint when Batch 3 was examined. This could reflect the selection of a variant that was able to escape the immune response and that was transmitted efficiently in the vaccinated population that had been infected or in contact with the initial one. One evident

explanation could have been the selection of a variant with enhanced replication or capacity of transmission. Our replication kinetics experiments however did not support the hypothesis of an enhanced replication capability, although we cannot discard higher potential for transmission, not caused by an increased yield of replication itself, but by a better replication in the nasal mucosa macrophages (Frydas and Nauwynck, 2016).

Interestingly, in Batch 3 the proportion of vertical transmission events was very high suggesting again that either immunity against the new variant was not strong enough in the population or that non-immune sow subpopulations existed. When we examined the NAb against the variant predominating in Batch 3, it was evident that even sows that had been infected (as demonstrated by the delivery of infected piglets) developed very low levels of NAb. This is an indication that the strain present in the farm was probably a poor inducer of NAb. Moreover, the fact that the offspring of old sows that had been vaccinated on multiple occasions and had given birth to infected piglets were negative in the neutralization tests indicates the vaccination did not contribute to enhance the humoral response of the sows against an unrelated strain. Furthermore, it is evident that subpopulations of susceptible sows can be found in multiply vaccinated farms with high circulation of wild type virus (Díaz et al., 2020; Fiers et al., 2022). In a small farm like the one studied here, after an outbreak and with vertical transmission rates of 30%, it seems unlikely that solely by chance so many sows did never encounter the wild-type virus and, in consequence, did not produce antibodies specific for the farm strain. It is tempting to think that they are unresponsive sows. This hypothesis would be supported by the observation that the offspring of sows that transmitted the infection at birth did not have Nabs, not even in the case of an old sow delivering infected piglets twice. These breeders are clear candidates to maintain infection on the farm. Elucidating the role of those poor responder sows and determining if the lack of response occurred from the initial immunization or was the result of receiving multiple vaccine doses would be important to understand how the virus may persist in vaccinated farms. However, the fact that the reproductive disease was under control after the initial outbreak, and no changes in the farm management or the vaccination schedule were performed, suggests that some immunity existed in sows. If NAb specific for the farm strain were low, the most logical explanation would be that cell-mediated immunity had a role in this control. However, the fact that in Batch 3 vertical transmission was high opens the question of the duration of the efficacious immunity after infection with some strains.

Taken together, in our opinion, the most likely scenario was as follows: the introduction of a new strain in the farm found a population that had limited immunity against that strain despite vaccination. At that moment, the infection spread rapidly, affected pregnant sows, and they transmitted the infection vertically to the offspring. This vertical transmission involved several bottlenecks (transmission through placenta, transmission to vaccinated sows) that generated, from the replication of founder variants, a higher diversity circulating in the piglets, a fact that suggested that the bottleneck selection had a component of probability derived from different phenomena (population bottlenecks, genetic bottlenecks, etc.). In a subsequent phase, the population became immune and limited the transmission to a few individuals. However, since the strain infecting the animals in the farm apparently was a poor inducer of immunity (low levels of NAb and rapid decay of these), susceptible subpopulations rapidly appeared in which the virus kept circulating until a fitter variant was selected and spread again in the population. At this point, all previous diversity would disappear and would be replaced by the new variant. An interesting question raises here, is it possible to identify beforehand what animals contribute to the maintenance of the virus in that phase of limited circulation? This is an area that needs to be explored in further studies.

Interestingly, when highly variable positions were examined within the viral quasi-species of animals in Batch 1 and 3, the number of highly variable positions decreased in Batch 3, suggesting that selection reduced the variability in those highly variable positions. Furthermore, in both Batch 1 and 3, a high proportion (30 to 55%) of the highly variable positions within the quasi-species were in nsp1a despite that nsp1a only accounts for roughly 3.5% of the viral genome. Nsp1a is involved in the downregulation of type 1 interferon (Beura et al., 2010; Chen et al., 2010; Beura et al., 2012). This activity has been located in the PCPα domain of the protein. Of note, almost all the highly variable positions in nsp1a were found outside that area, in the zinc-finger motif, suggesting that variability was not related with the inhibition of the interferon response. Besides, nsp1a has been shown to contain at least two epitopes inducing IFN-gamma responses in CD8 T cells (Mötz et al., 2022). One of the most highly variable positions affected one of them but it is difficult to predict the result of this variability on the effective immunity against the virus. Moreover, nsp1a is involved in the induction of apoptosis, pro-inflammatory responses, and the regulation of minus strand templates (Nedialkova et al., 2010; Wang et al., 2016;

Park et al., 2021) and thus the variability may respond to other functionalities of the protein.

The present study also provides some relevant information about transmission. The identification of animals, viral variants, and movements between pens allowed to determine, at least partially, the transmission chains. These data showed that while most infected animals contributed to 1–3 transmissions, some animals apparently did not transmit the infection to anyone else, and one (animal 502) was identified as the source of 8 cases out of 28 horizontal transmission events (28.6%) behaving like a super-spreader. Whether this superspreading capability derived from host characteristics, environmental conditions, or both, cannot be determined. It was recently suggested that superspreading events can be an intrinsic characteristic of each viral infection (Chen et al., 2021). It is not known if different viral variants may be more prone to produce superspreading events, but, in our opinion, this would not be the case in our study since other animals infected by the same variant transmitted the infection only to one susceptible recipient.

Another surprising observation was the fact that one animal that was born-viremic and persisted as so for the whole length of the study, apparently did not contribute to the transmission of the virus. In general, it is thought that animals infected with PRRSV in utero are important spreaders due to the long viremias they develop (Rowland et al., 1999; Benfield et al., 2000; Rowland et al., 2003). However, the evidence provided by this study indicates that, at least in some cases, these animals may not contribute significantly to the transmission. It would be interesting to examine the shedding of the virus in oral fluids and nasal secretions of these born-viremic animals compared to animals infected after birth to determine differences in transmission capabilities.

From the practical point of view, it is also worth commenting the results of Batch 2. Commonly, PRRSV stability is determined after the examination by RT-qPCR of a number of animals at weaning (usually 30 to 60 animals at wean, in four consecutive batches; Holtkamp, 2011). In our case, only one sample out of 62 (1.6%) was positive at weaning with a Ct of 31.9. This suggests that in endemic situations sampling to determine stability of a herd should be of at least 60 animals, and probably more. The low prevalence at weaning in that batch was enough to allow the reemergence of the virus some weeks later.

3.6. CONCLUSION

The present paper reports an example of how PRRSV may persist in a vaccinated farm. Upon introduction of a new strain, founder effects related to transplacental transmission and high rates of horizontal transmission produce a high diversity of viral variants, but it simultaneously generates an immune or partially immune population, resulting in a low-level circulation of the virus. The combination of subpopulations that just by chance did not have contact with the virus together with a viral strain that was a poor inducer of long-lasting immunity, kept the virus circulating at a low level. Nevertheless, this was enough to allow the selection of a variant that became predominant afterwards. The role of super-spreaders and long-term viremic animals must be re-examined after the results of the present study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was reviewed and approved by Ethics Committee in Animal and Human Research of the Universitat Autònoma de Barcelona. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MM, MC, and EM designed the study. Laboratory work was performed by HC, YL, GM-V, MC, and EM. EM, MC, GM-V, YL, and HC analyzed the results. EM, MC, and HC prepared the draft of the manuscript. All authors have read, revised, discussed, and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.7. SUPPLEMENTARY MATERIAL

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1109881/full#supplementary-material

Table S1. List of oligonucleotide primers (F: forward primer, R: reverse primer) used for amplifying PRRSV-1 ORF5, nsp2, and nsp9 in this study and the size of the amplicon.

Gene		Sequence	Amplicon (bp)
ORF5			
	L1F (F)	5′-TGAGGTGGGCTACAACCATT-3′	702
	L1R (R)	5′-AGGCTAGCACGAGCTTTTGT-3′	
nsp2			
	nsp2-F1 (F)	5′-AGGAGTTTTGGACCCTTGACA-3’	617
	nsp2-R1 (R)	5′-GGGTCAARTCCAGTGGTTC-3’	
	nsp2-F2 (F)	5′-ACAGAGACTTGCCCTCCTCA-3’	643
	nsp2-R2 (R)	5′-GCACTGTTCATATACCCGGC-3’	
	nsp2-F3 (F)	5′-GCCGGGTATATGAACAGTGC-3’	678
	nsp2-R3 (F)	5′-AAAACACCCAGACGAACACG-3’	
nsp9			
	nsp9-F (F)	5’-TGACCACTGAACAGGCTTTAAACT-3’	684
	nsp9-R (R)	5’-CATAAAAGGAGTGTCAGGGCG-3’	

Table S2. List of PRRSV-1 complete genome sequences retrieved from GenBank used for the phylogenetic analysis. Lelystad virus is the prototype for PRRSV-1. Contemporary strains within the same geographical zone of the farm (underlined) and the 5 commercially licensed vaccines in Spain (in red) are included.

Accession Number	Strain ID	Country	Year
NC043487	***Lelystad virus***	Netherlands	1993
KF203132	*Olot/91*	Spain	1991
JF276431	*CReSA3262*	Spain	1992
KX249748	*CReSA3*	Spain	2013
KX249755	*CReSA228*	Spain	2013
KX249756	*CReSA261*	Spain	2013
KX249753	*CReSA100*	Spain	2014
OP822977	*Nu4a*	Spain	2021
OP822964	*M3*	Spain	2021
OP822963	*N5*	Spain	2021
OP822973	*Nu1*	Spain	2021
OM893828	*R1*	Spain	2021
OM893829	*R2*	Spain	2021
JF276435	*CReSA3267*	Portugal	2006
KT326148	*AUT13-883*	Austria	2013
JF802085	*Lena*	Belarus	2007
KP889243	*SU1-Bel*	Belarus	2010
GU737264	*07V063*	Belgium	2007
KT159248	*13V091*	Belgium	2013
KT159249	*13V117*	Belgium	2013
GU047344	*BJEU06-1*	China	2006
MN927227	*HeB3*	China	2018
KC862567	*DK-2011-0511-14*	Denmark	2011
KY767026	*FR-2014-56-11-1*	France	2014
MH018883	*FR-2016-56-11-1*	France	2016
MH463457	*HU19401*	Hungary	2016
MF346695	*PR40-2014*	Italy	2014
KX668221	*WestSib13*	Russia	2013
KY434183	*CBNU0495*	South Korea	2016
GU067771	***Unistrain®PRRS***	-	-
KJ127878	***Porcilis® PRRS***	-	-
LQ787782	***Suvaxyn® PRRS***	-	-
GQ461593	***PYRSVAC-183®***	-	-
KT988004	***PRRSFlex®EU***	-	-

Figure S1. Bayesian phylogenetic tree based on complete genomes of PRRSV-1 using MrBayes (1,000,000 iterations). The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. Lelystad strain is the prototype for PRRSV-1. The red colored strains are the vaccines commercially licensed in Spain. The underlined strains are contemporary strains within the same geographical zone of the farm. Only posterior probability values >70% are shown.

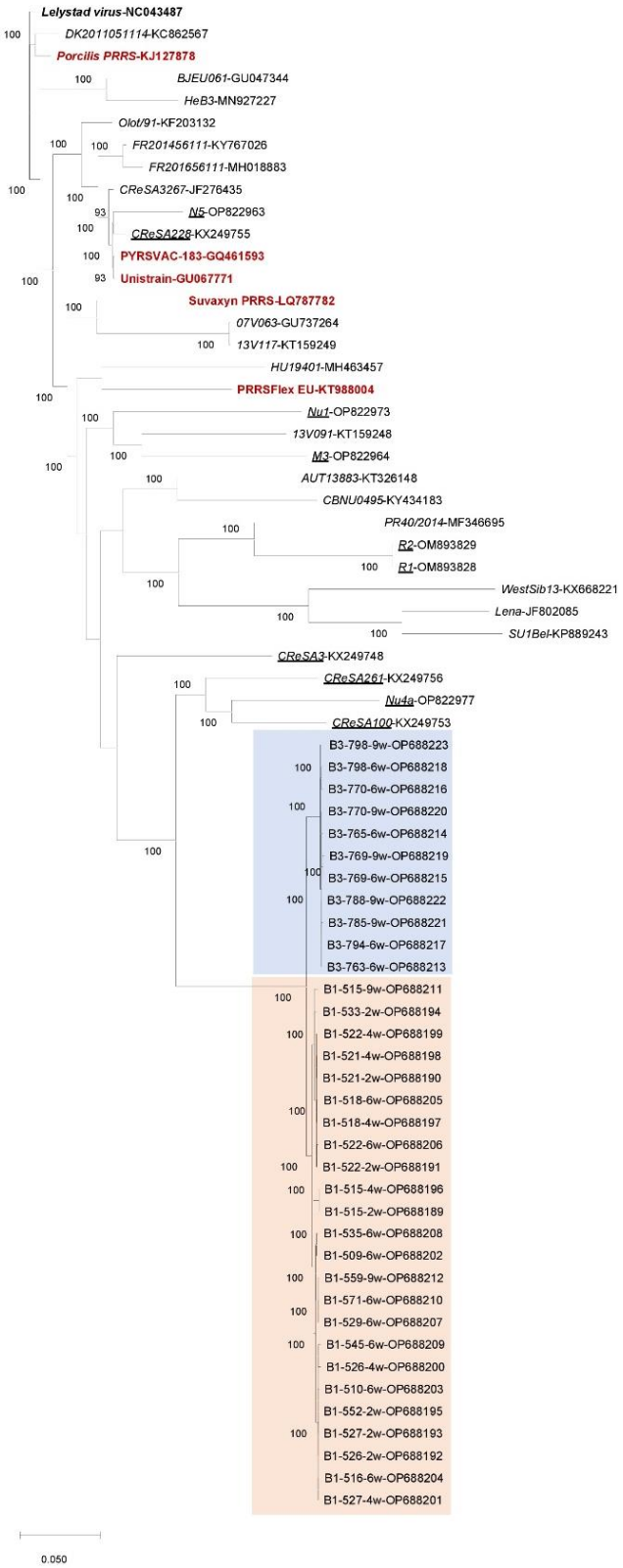


Figure S2. Bayesian phylogenetic tree based on PRRSV-1 ORF5 sequences using MrBayes (1,000,000 iterations). The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. Lelystad strain is the prototype for PRRSV-1. The red colored strains are the vaccines commercially licensed in Spain. The underlined strains are contemporary strains within the same geographical zone of the farm. Only posterior probability values >70% are shown.

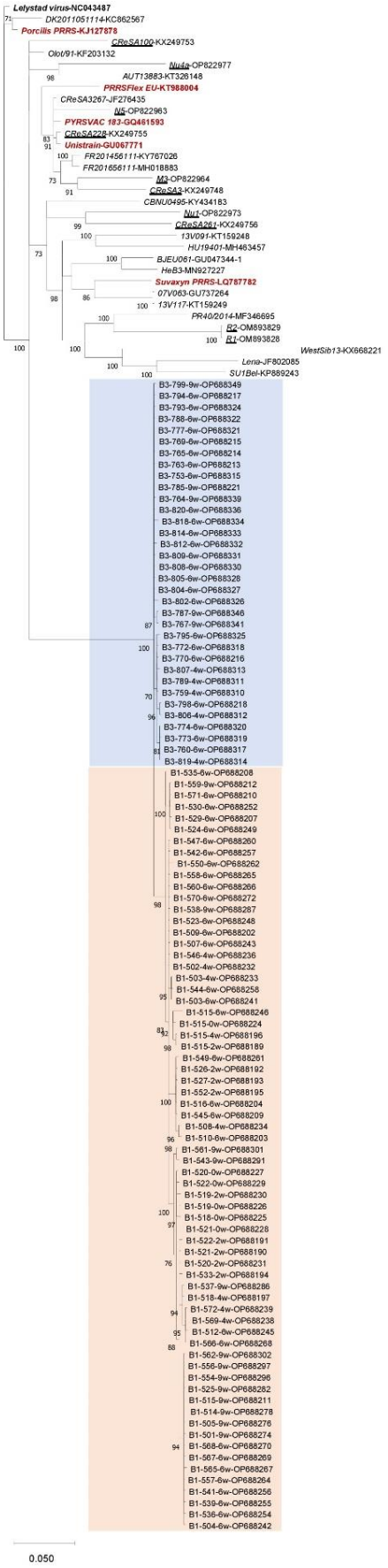


Table S3. Similarities (p-distance) between the circulating virus in this study and the reference PRRSV-1 strains. The table shows the nucleotide mean differences between the examined batches of PRRSV-1 whole genome consensus and ORF sequences and the sequences retrieved from GenBank.

	Genome		ORF5	
PRRSV-1 strain	Batch 1	Batch 3	Batch 1	Batch 3
***Lelystad virus*-NC043487**	0,1353	0,1374	0,1421	0,1403
Olot/91-KF203132	0,1347	0,1359	0,1484	0,1469
CReSA3-KX249748	0,1678	0,1693	0,1535	0,1521
CReSA228-KX249755	0,1347	0,1361	0,1441	0,1421
CReSA261-KX249756	0,1333	0,1341	0,1808	0,1799
CReSA100-KX249753	0,1411	0,1420	0,1720	0,1700
Nu4a-OP822977	0,1583	0,1596	0,1814	0,1799
M3-OP822964	0,1762	0,1789	0,1619	0,1601
N5-OP822963	0,1508	0,1520	0,1573	0,1568
Nu1-OP822973	0,1769	0,1776	0,1792	0,1782
R1-OM893828	0,1945	0,1953	0,1773	0,1766
R2-OM893829	0,1944	0,1952	0,1773	0,1766
CReSA3267-JF276435	0,1338	0,1350	0,1423	0,1403
AUT13883-KT326148	0,1755	0,1779	0,1611	0,1601
Lena-JF802085	0,2126	0,2138	0,2000	0,1997
SU1Bel-KP889243	0,2126	0,2131	0,1830	0,1832
07V063-GU737264	0,1643	0,1659	0,1528	0,1518
13V091-KT159248	0,1759	0,1773	0,1970	0,1980
13V117-KT159249.1	0,1644	0,1659	0,1528	0,1518
BJEU061-GU047344.1	0,1589	0,1610	0,1658	0,1667
HeB3-MN927227	0,1588	0,1605	0,1562	0,1551
DK2011051114-KC862567	0,1407	0,1427	0,1477	0,1471
FR201456111-KY767026	0,1340	0,1355	0,1475	0,1452
FR201656111-MH018883	0,1379	0,1396	0,1475	0,1452
HU19401-MH463457	0,1726	0,1737	0,2066	0,2063
PR40/2014-MF346695	0,1862	0,1859	0,1817	0,1815
WestSib13-KX668221	0,2171	0,2178	0,2111	0,2112
CBNU0495-KY434183	0,1738	0,1749	0,1646	0,1634
***Porcilis PRRS*-KJ127878**	0,1352	0,1370	0,1438	0,1436
***PRRSFlex EU*-KT988004**	0,1610	0,1625	0,1417	0,1403
***Unistrain*-GU067771**	0,1339	0,1353	0,1456	0,1436
***Suvaxyn PRRS*-LQ787782**	0,1518	0,1544	0,1665	0,1650
***PYRSVAC183*-GQ461593**	0,1341	0,1354	0,1472	0,1452

Figure S3. Bayesian phylogenetic tree based on the partial nucleotide sequences of PRRSV-1 nsp2 using MrBayes (1,000,000 iterations). nsp2 sequences obtained from born-infected animals with long viremias (in red) and the new infection cases (in black) are included. The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. The shaded sequences with the same color correspond to a transmission chain. Only posterior probability values >70% are shown.

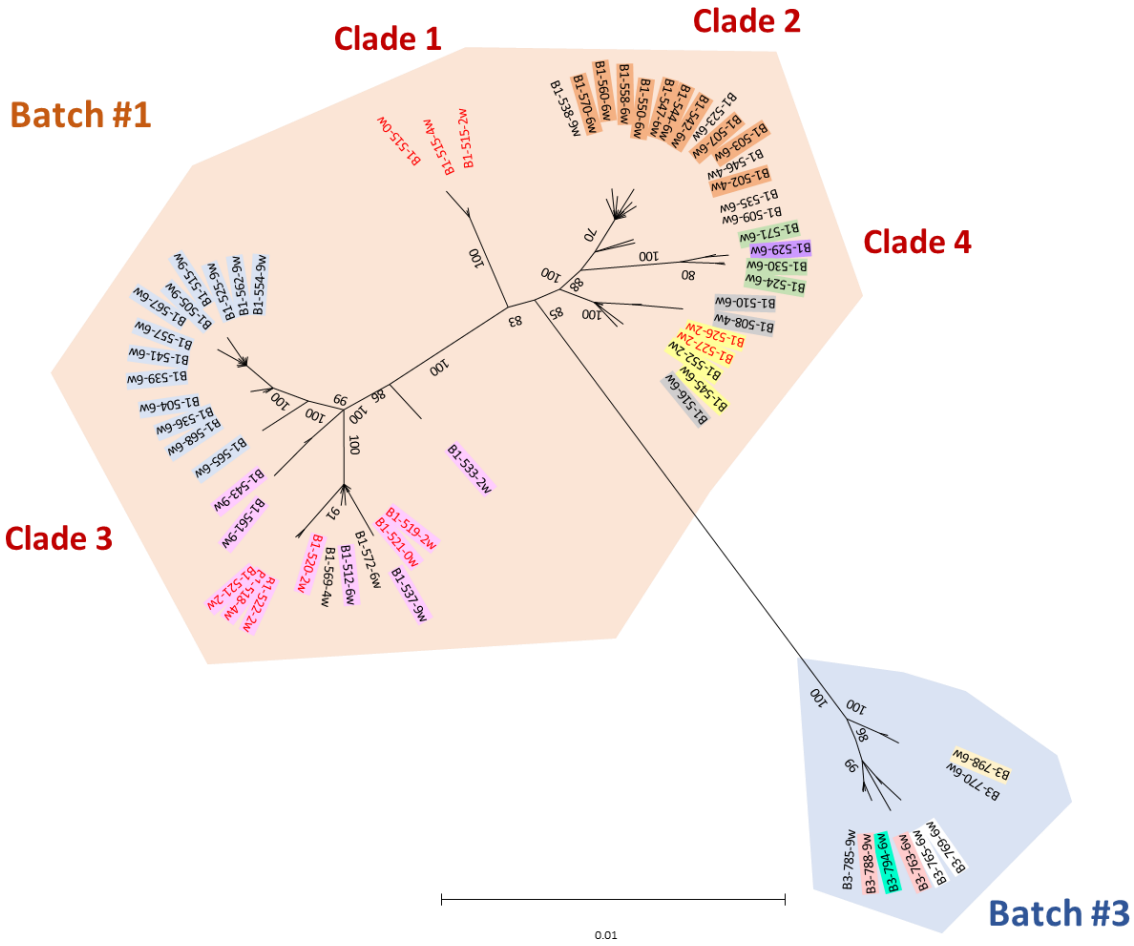


Figure S4. Bayesian phylogenetic tree based on the partial nucleotide sequences of PRRSV-1 nsp9 using MrBayes (1,000,000 iterations). nsp9 sequences obtained from born-infected animals with long viremias (in red) and the new infection cases (in black) are included. The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. The shaded sequences with the same color correspond to a transmission chain. Only posterior probability values >70% are shown.

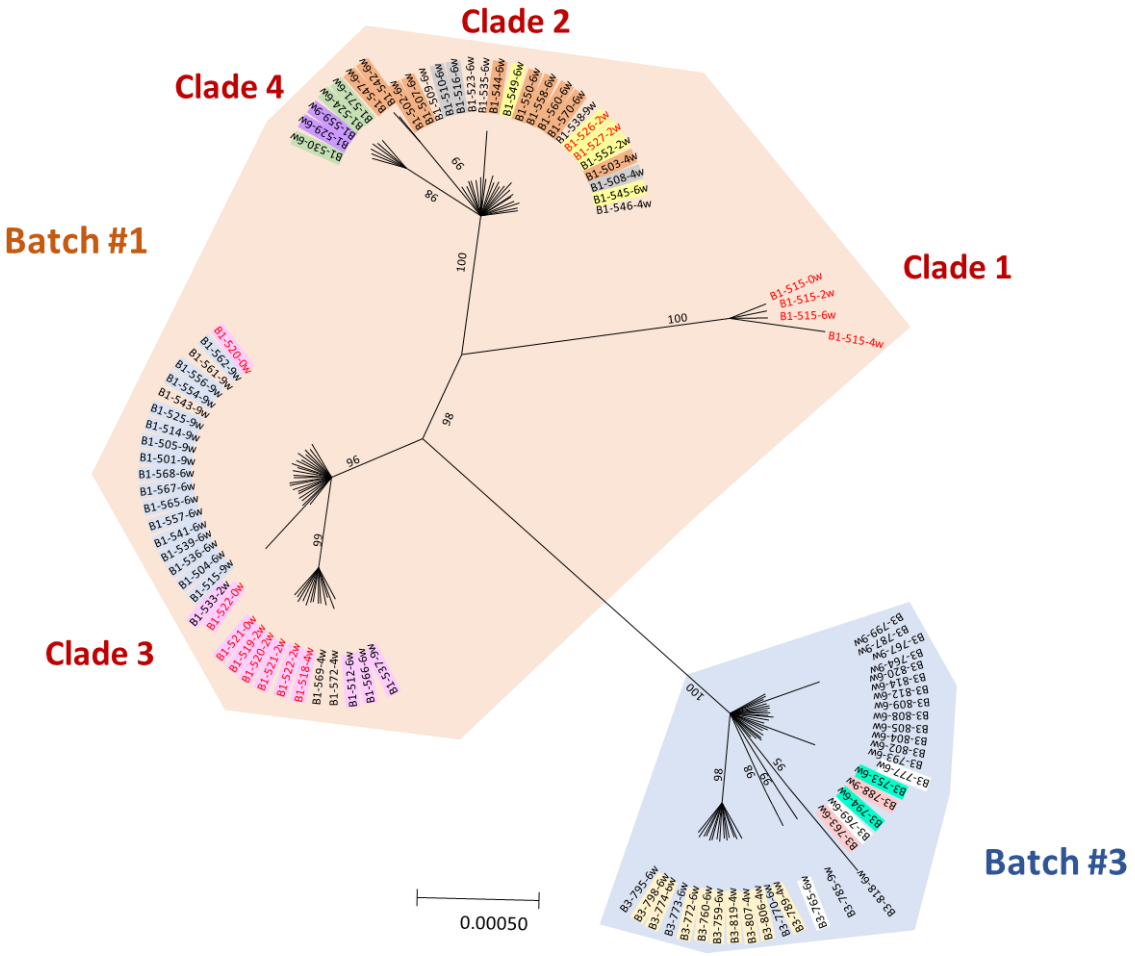


Figure S5. Bayesian phylogenetic tree based on the whole genome nucleotide sequences of PRRSV-1 using MrBayes (1,000,000 iterations). Whole genome sequences obtained from born-infected animals with long viremias (in red) and the new infection cases (in black) are included. The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. The shaded sequences with the same color correspond to a transmission chain. Only posterior probability values >70% are shown.

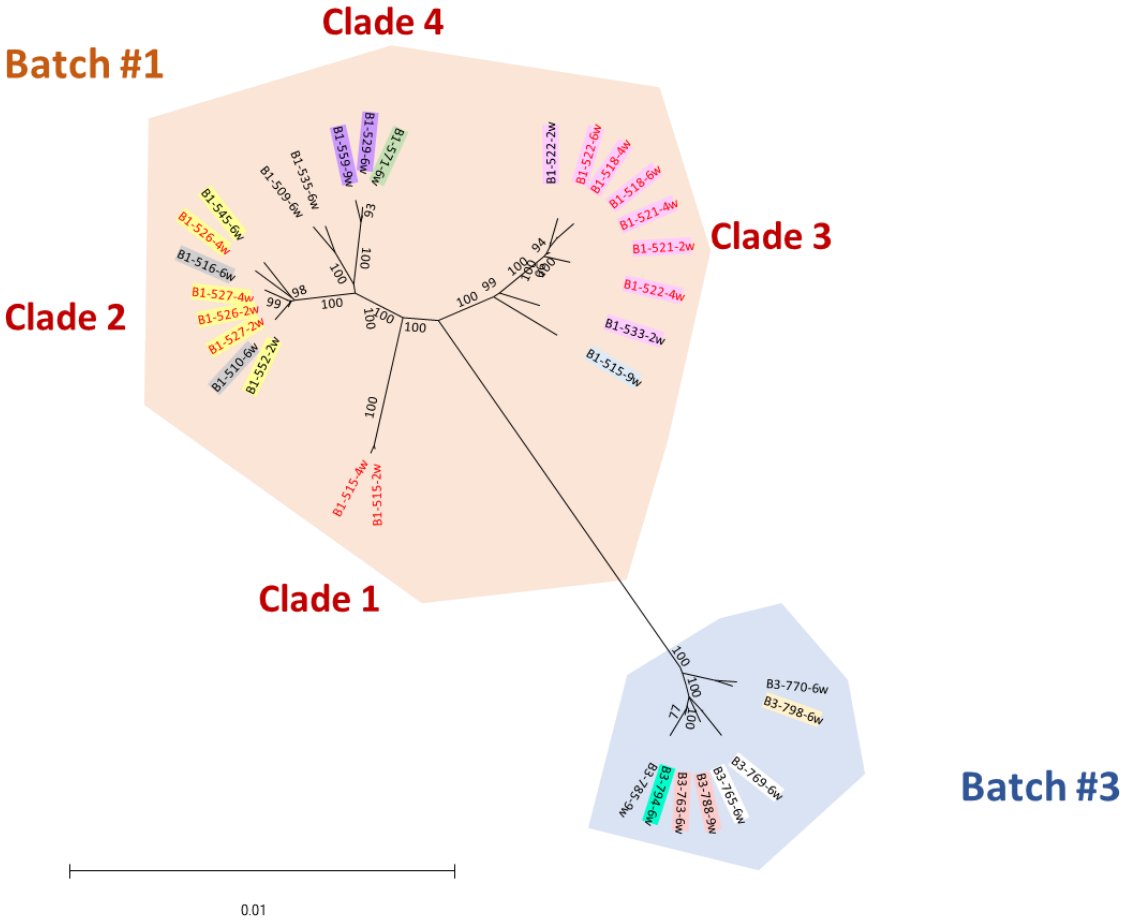


Figure S6. Maximum likelihood phylogenetic tree based on the ORF5 nucleotide sequences of PRRSV-1. Whole genome sequences obtained from born-infected animals with long viremias (in red) and the new infection cases (in black) are included. The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. The shaded sequences with the same color correspond to a transmission chain. Only bootstrap values >60% are shown.

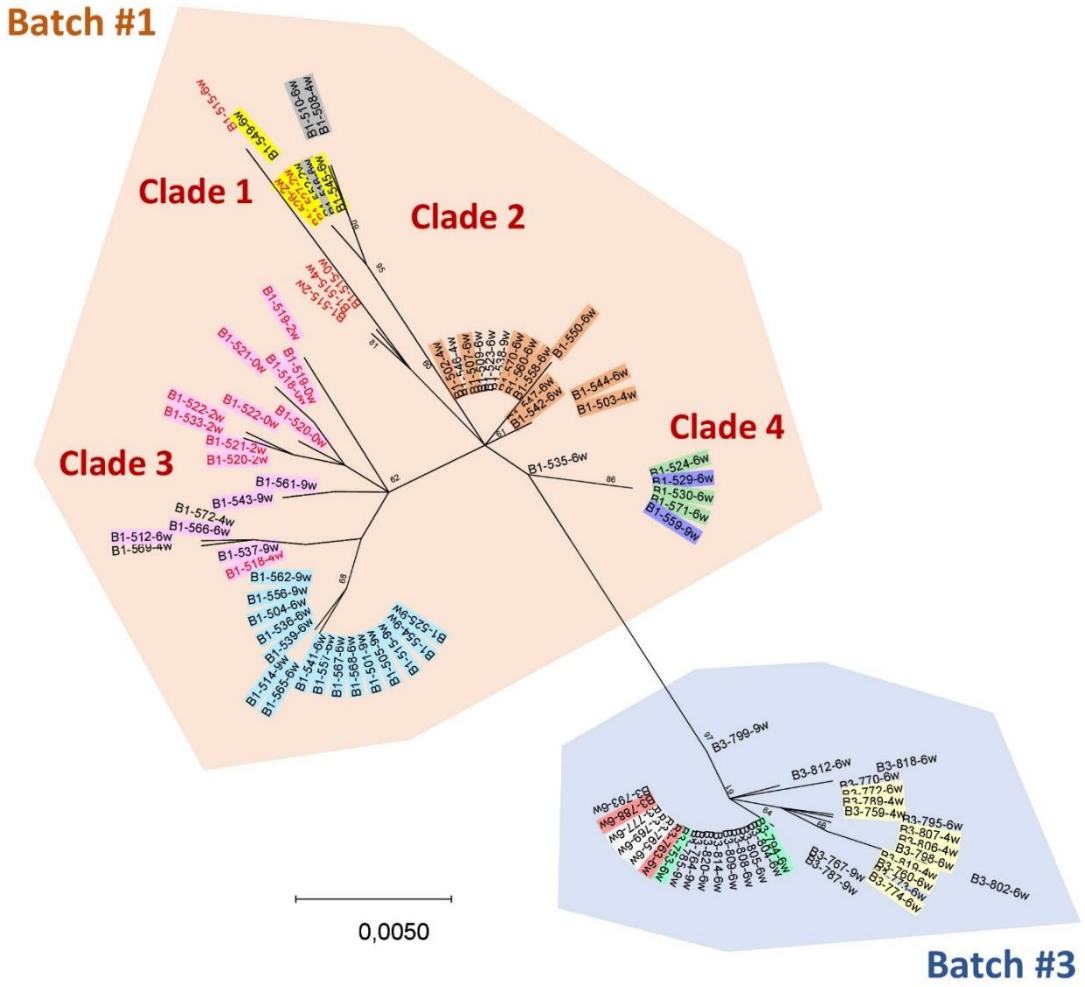


Figure S7. Maximum likelihood phylogenetic tree based on the partial nsp2 nucleotide sequences of PRRSV-1. Whole genome sequences obtained from born-infected animals with long viremias (in red) and the new infection cases (in black) are included. The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. The shaded sequences with the same color correspond to a transmission chain. Only bootstrap values >60% are shown.

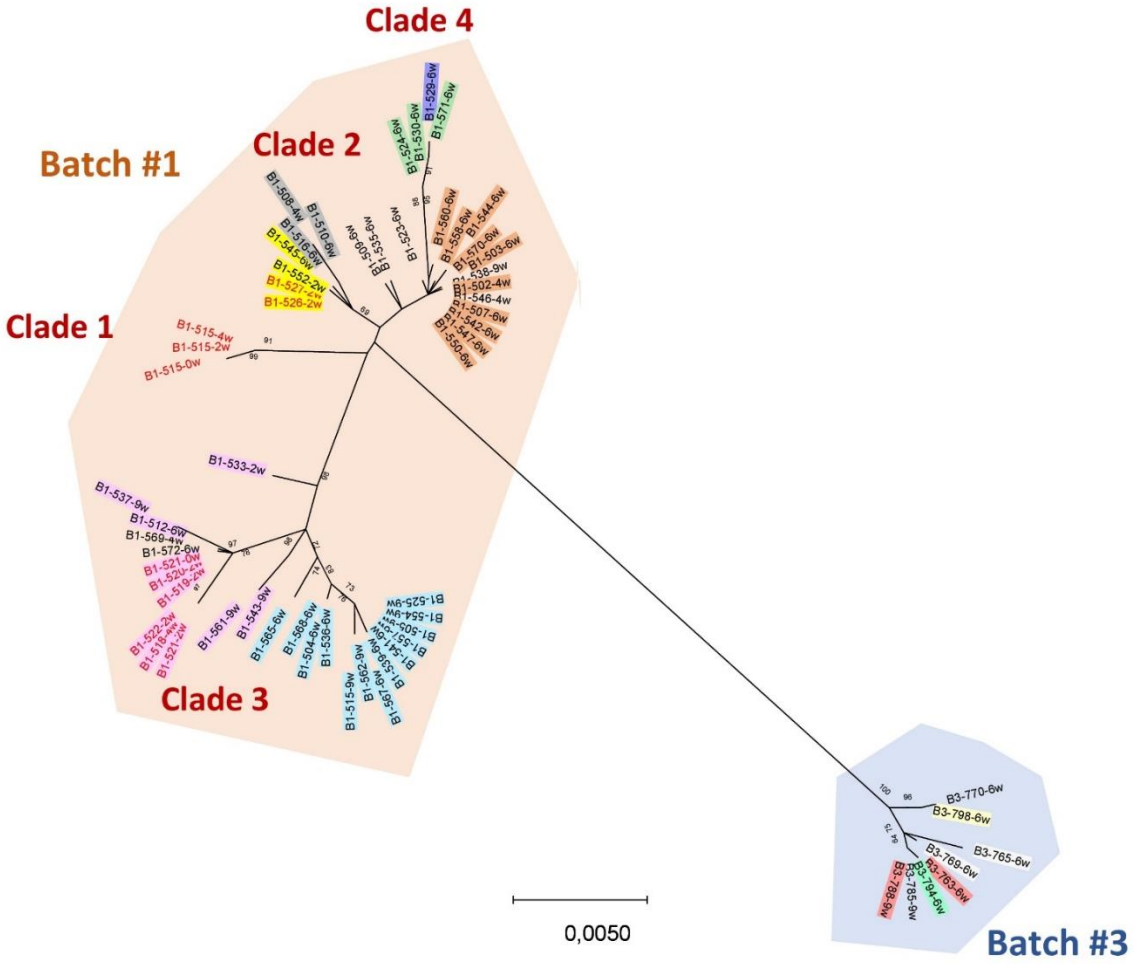


Figure S8. Maximum likelihood phylogenetic tree based on the partial nsp9 nucleotide sequences of PRRSV-1. Whole genome sequences obtained from born-infected animals with long viremias (in red) and the new infection cases (in black) are included. The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. The shaded sequences with the same color correspond to a transmission chain. Only bootstrap values >60% are shown.

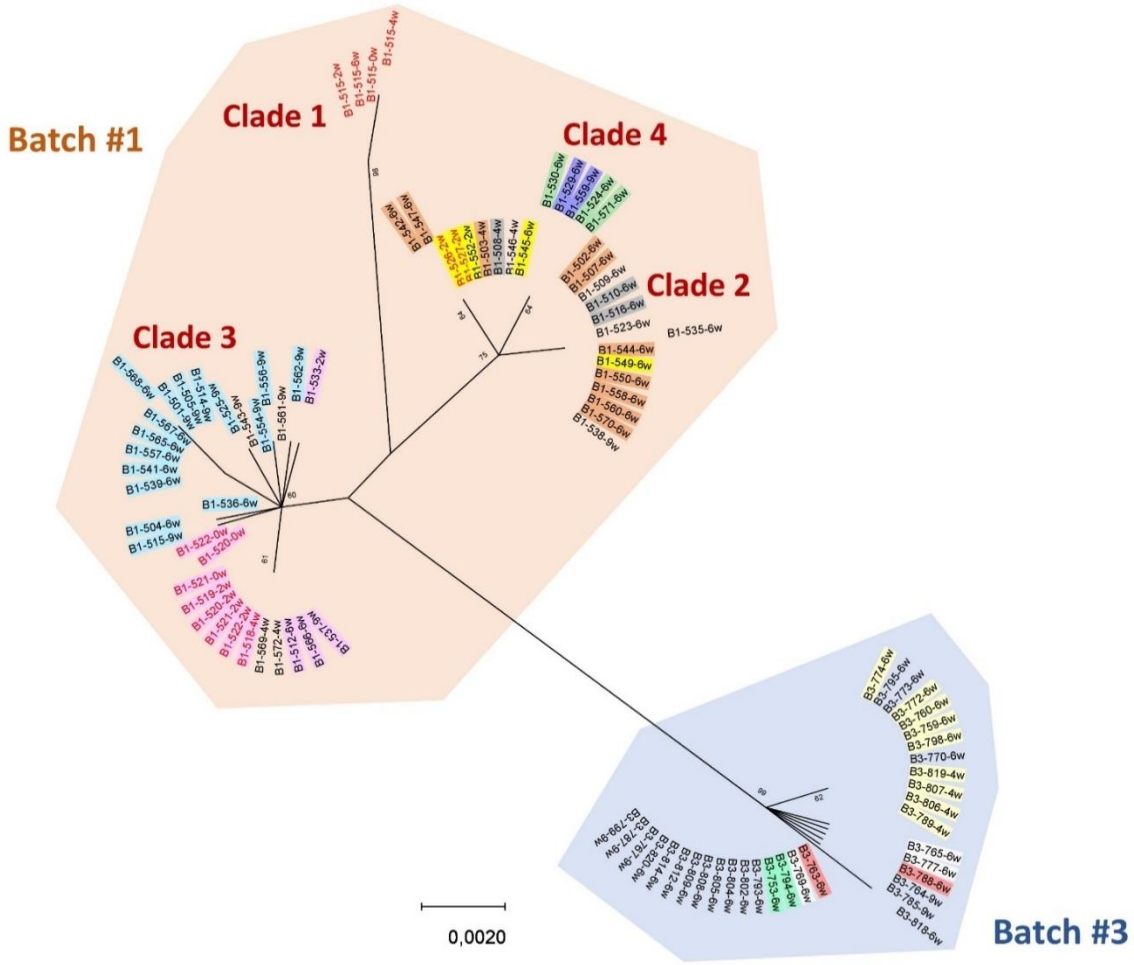


Figure S9. Maximum likelihood phylogenetic tree based on the whole genome nucleotide sequences of PRRSV-1. Whole genome sequences obtained from born-infected animals with long viremias (in red) and the new infection cases (in black) are included. The orange and blue shaded areas correspond to sequences retrieved from Batch 1 and 3, respectively. The shaded sequences with the same color correspond to a transmission chain. Only bootstrap values >60% are shown.

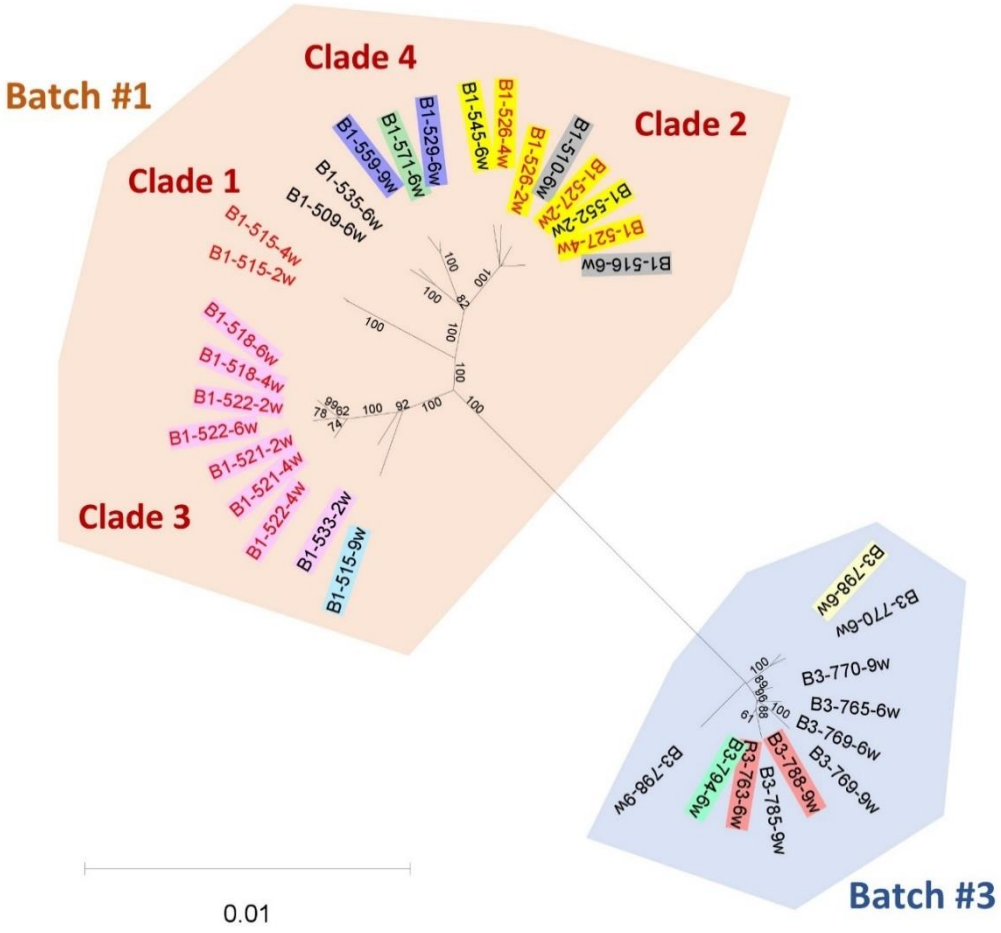


Table S4. Amino acid comparison of PRRSV-1 sequences between batches and clades. The table shows the differences between the predicted amino acid composition of the different viral proteins inferred from the consensus nucleotide sequences for each detected clade of Batch 1 (B1) and 3 (B3) (in rows). Columns indicate positions where amino acid differences were found in each examined protein. The amino acid position corresponds to the alignment with the prototype PRRSV-1 strain Lelystad (LV; NC_043487). For nsp2, all detected clades presented a 5-aa deletion between position 347-351 referred to LV. In that case, the number between brackets indicated the position considering the deletion. Grey shaded cells show the position where a different amino acid was found. Red-shaded positions indicate variations affecting known neutralizing epitopes. Changes affecting known glycosylation sites are marked with a blue circle.

	nsp1a					nsp1b								nsp2								
Amino acid position	43	80	127	130	151	8	16	28	72	80	81	117	178	26	174	215	269	287	291	295	303	316
B1-Clade1	G	T	G	P	P	K	P	G	H	H	F	G	R	Q	T	Q	P	A	P	K	E	V
B1-Clade2	G	T	S	P	P	E	P	G	H	H	S	D	H	Q	T	Q	P	A	P	K	E	V
B1-Clade3	G	V	S	P	P	E	T	G	H	H	S	G	R	K	T	Q	P	A	L	N	E	V
B1-Clade4	G	T	S	P	P	E	P	G	H	H	S	D	R	Q	T	R	P	A	P	K	E	V
B3	D	A	S	S	S	E	P	V	R	Y	S	G	R	Q	N	Q	L	T	P	K	K	M

	nsp2																	
Amino acid position	336	342	347-351	368 (363)	370 (365)	372 (367)	377 (372)	378 (373)	379 (374)	388 (383)	390 (385)	395 (390)	396 (391)	402 (397)	404 (399)	415 (410)	418 (413)	434 (429)
B1-Clade1	K	L	Del	L	S	E	S	D	W	R	A	I	T	T	G	V	Q	N
B1-Clade2	K	L	Del	L	S	E	N	D	W	Q	A	I	T	T	G	A	Q	S
B1-Clade3	K	L	Del	L	S	G	N	D	W	R	A	T	T	T	G	V	Q	S
B1-Clade4	K	L	Del	L	S	E	N	D	W	R	A	I	A	T	G	A	Q	S
B3	R	V	Del	P	P	E	N	N	R	R	T	I	T	A	S	A	R	S

	nsp2																	
Amino acid position	439 (434)	440 (435)	451 (446)	482 (477)	509 (504)	511 (506)	520 (515)	521 (516)	541 (536)	545 (540)	567 (562)	645 (640)	677 (672)	689 (684)	698 (693)	711 (706)	712 (707)	714 (709)
B1-Clade1	N	R	T	V	I	S	D	D	L	K	E	I	S	S	L	R	M	T
B1-Clade2	D	R	T	V	I	S	D	D	L	K	E	I	S	P	L	R	M	T
B1-Clade3	D	R	A	V	V	S	D	D	L	K	E	I	L	P	F	R	V	A
B1-Clade4	D	R	T	V	I	S	D	D	L	K	A	I	D	S	L	R	M	T
B3	D	G	T	I	I	F	N	G	F	T	E	T	D	P	L	C	M	T

	nsp2				nsp3			nsp4						nsp6	nsp7a	nsp9				
Amino acid position	719 (714)	728 (723)	830 (825)	957 (952)	59	61	77	33	72	147	163	172	188	14	145	23	106	109	160	221
B1-Clade1	A	V	L	S	S	S	A	T	D	D	K	L	I	S	T	E	N	I	R	V
B1-Clade2	A	A	L	S	P	S	A	T	E	N	R	L	I	S	T	G	D	I	R	M
B1-Clade3	A	A	L	S	S	S	V	I	D	N	K	L	I	N	T	E	D	T	R	M
B1-Clade4	T	A	L	S	P	S	A	T	E	N	R	L	V	S	T	G	D	I	R	M
B3	A	A	F	P	S	P	A	I	D	N	K	I	I	S	I	E	D	I	C	M

	nsp9	nsp10			nsp11				nsp12			GP2							E	GP3		
Amino acid position	618	12	66	428	124	139	155	182	58	59	147	8	19	78	83	197	224	236	69	18	22	30
B1-Clade1	Q	A	T	L	A	T	S	I	H	I	P	V	L	N	I	K	M	V	V	H	G	S
B1-Clade2	Q	T	T	P	A	T	S	V	H	V	P	V	L	N	V	R	I	A	I	H	S	F
B1-Clade3	Q	A	T	P	A	T	S	V	Y	V	P	V	L	N	V	K	I	V	V	Y	S	F
B1-Clade4	Q	A	T	P	A	T	S	V	H	V	P	V	P	D	V	K	I	A	V	H	S	F
B3	R	A	A	P	V	A	F	V	H	I	L	A	L	N	V	K	I	V	V	Y	S	F

	GP3					GP4					GP5							M					
Amino acid position	84	91	220	239	242	45	65	69	77	145	8	41	46	56	59	106	182	3	6	12	28	68	69
B1-Clade1	G	S	P	S	R	M	G	K	V	Y	E	R	D	S	S	E	V	S	D	A	M	N	R
B1-Clade2	G	L	P	S	H	M	S	K	V	Y	E	R	D	S	S	G	V	N	D	T	M	D	R
B1-Clade3	V	S	L	F	R	M	S	K	I	Y	E	R	N	S	Y	G	I	S	D	T	M	N	R
B1-Clade4	E	S	P	S	R	M	S	K	V	Y	E	R	N	S	S	G	V	S	D	T	M	N	R
B3	E	S	P	F	R	V	S	E	I	H	G	Y	N	F	S	E	V	S	G	T	I	N	Q

	M		N	
Amino acid position	73	130	51	127
B1-Clade1	I	Q	P	A
B1-Clade2	T	R	P	E
B1-Clade3	I	R	L	A
B1-Clade4	T	R	P	A
B3	T	R	P	A

Table S5. Amino acid comparison of PRRSV-1 sequences between the circulating virus and the vaccine strain used in the farm. The table shows the differences between the vaccine strain and the predicted amino acid composition of the different viral proteins inferred from the consensus nucleotide sequences of Batch 1 (B1) and 3 (B3) (in rows). Columns indicate positions where amino acid differences were found in each examined protein. The amino acid position corresponds to the alignment with the prototype PRRSV-1 strain Lelystad (LV; NC_043487). For nsp2, all detected clades presented a 5-aa deletion between position 347-351 referred to LV. In that case, the number between brackets indicated the position considering the deletion. Red-shaded positions indicate variations affecting known neutralizing epitopes.

nsp1a (n=20)

Amino acid position	42	43	45	47	54	57	58	80	81	100	113	117	130	138	139	140	141	148	151	162
Vaccine	**T**	**D**	**G**	**V**	**R**	**L**	**H**	**A**	**V**	**K**	**P**	**C**	**P**	**M**	**G**	**L**	**F**	**F**	**P**	**L**
B1	P	G	A	I	K	I	S	V/T	I	R	S	R	P	I	A	V	Y	S	P	S
B3	P	D	A	I	K	I	S	A	I	R	S	R	S	I	A	V	Y	S	S	S

nsp1b (n=57)

Amino acid position	1	7	8	9	15	16	17	18	21	26	28	30	33	34	37	40	42	43	44	50	58	59	62	63
Vaccine	**F**	**Q**	**E**	**F**	**F**	**P**	**I**	**D**	**S**	**T**	**G**	**S**	**A**	**G**	**E**	**G**	**I**	**R**	**C**	**I**	**F**	**A**	**T**	**E**
B1	A	R	E/K	Y	S	P/T	S	G	P	S	G	D	V	A	V	P	L	E	H	T	P	V	A	D
B3	A	R	E	Y	S	P	S	G	P	S	V	D	V	A	V	P	L	E	H	T	P	V	A	D

Amino acid position	66	67	72	77	78	80	84	86	91	94	99	102	103	104	105	106	109	110	111	114	117	118
Vaccine	**F**	**T**	**R**	**N**	**T**	**H**	**H**	**V**	**V**	**G**	**S**	**L**	**G**	**Q**	**S**	**A**	**R**	**C**	**H**	**H**	**D**	**A**
B1	L	A	H	G	V	H	Y	A	I	S	T	F	D	L	P	T	W	R	R	Y/C	G/D	S
B3	L	A	R	G	V	Y	Y	A	I	S	T	F	D	L	P	T	W	r	R	Y	G	S

Amino acid position	138	139	141	169	170	178	180	193	194	195	200
Vaccine	**V**	**H**	**I**	**N**	**D**	**R**	**T**	**S**	**R**	**I**	**A**
B1	I	N	V	D	S	R/H	M	L	P	L	V
B3	I	N	V	D	S	R	M	L	P	L	V

nsp2 (n=197)

Amino acid position	11	15	20	21	23	24	25	26	28	30	32	33	57	66	67	68	82	93	94	107	120	128	141
Vaccine	**A**	**E**	**P**	**T**	**K**	**V**	**A**	**L**	**V**	**T**	**G**	**I**	**I**	**T**	**Q**	**Y**	**V**	**T**	**V**	**I**	**S**	**P**	**V**
B1	V	A	A	A	E	A	V	Q/K	T	A	R	T	V	A	P	F	A	A	I	V	P	S	T
B3	V	A	A	A	E	A	V	Q	T	A	R	T	V	A	P	F	A	A	I	V	P	S	T

Amino acid position	143	147	148	152	155	174	179	190	200	212	215	225	238	239	242	256	262	267	269	272
Vaccine	**P**	**A**	**D**	**K**	**L**	**A**	**N**	**K**	**S**	**V**	**Q**	**M**	**F**	**K**	**I**	**P**	**G**	**D**	**P**	**F**
B1	S	Q	N	T	P	T	D	R	P	I	Q/R	V	S	E	M	S	E	A	P	S
B3	S	Q	N	T	P	N	D	R	P	I	Q	V	S	E	M	S	E	A	L	S

Amino acid position	274	276	277	279	280	281-354 (281-349)	356 (351)	357 (352)	358 (353)	359 (354)	361 (356)	362 (357)	363 (358)	364 (359)	366 (361	367 (362)	368 (363)
Vaccine	**P**	**S**	**P**	**R**	**P**	**Del**	**G**	**L**	**I**	**N**	**V**	**G**	**G**	**N**	**S**	**P**	**S**
B1	S	F	Q	K	S		N	P	V	D	T	D	R	D	P	S	L
B3	S	F	Q	K	S		N	P	V	D	T	D	R	D	P	S	P

Amino acid position	369 (364)	370 (365)	372 (367)	375 (370)	376 (371)	377 (372)	378 (373)	379 (374)	380 (375)	381 (376)	387 (382)	388 (383)	390 (385)	395 (390)	399 (394)	400 (395)
Vaccine	**D**	**S**	**K**	**M**	**L**	**N**	**S**	**R**	**E**	**D**	**S**	**Q**	**A**	**T**	**R**	**E**
B1	G	S	E/G	T	P	N/S	D	W	G	E	T	R/Q	A	I/T	G	G
B3	G	P	E	T	P	N	N	R	G	E	T	R	T	I	G	G

Amino acid position	402 (397)	404 (399)	405 (400)	407 (402)	409 (404)	412 (407)	414 (409)	415 (410)	418 (413)	422 (417)	424 (419)	426 (421)	427 (422)	429 (424)	432 (427)	434 (429)
Vaccine	**T**	**D**	**N**	**G**	**D**	**A**	**P**	**V**	**R**	**P**	**G**	**I**	**L**	**H**	**H**	**G**
B1	T	G	I	N	G	V	S	A/V	Q	T	K	A	P	L	R	S
B3	A	S	I	N	G	V	S	A	R	T	K	A	P	L	R	S

Amino acid position	435 (430)	436 (431)	437 (432)	439 (434)	440 (435)	441 (436)	442 (437)	446 (441)	448 (443)	451 (446)	452 (447)	466 (461)	482 (476)	484 (479)	488 (483)	496 (491)
Vaccine	**T**	**E**	**S**	**D**	**S**	**S**	**S**	**Q**	**D**	**T**	**L**	**R**	**V**	**V**	**N**	**A**
B1	A	G	L	D/N	R	D	L	L	V	T/A	S	K	V	A	G	V
B3	A	G	L	D	G	D	L	L	V	T	S	K	I	A	G	V

Amino acid position	502 (497)	507 (502)	509 (504)	511 (506)	516 (511)	519 (514)	520 (515)	521 (516)	522 (517)	535 (530)	537 (532)	539 (534)	540 (535)	541 (536)	542 (537)	544 (539)
Vaccine	**L**	**P**	**I**	**F**	**D**	**V**	**V**	**D**	**A**	**V**	**N**	**F**	**K**	**F**	**A**	**K**
B1	F	S	I/V	S	G	A	D	D	V	A	D	L	E	L	V	T
B3	F	S	I	F	G	A	N	G	V	A	D	L	E	F	V	T

Amino acid position	563 (558)	567 (562)	570 (565)	592 (587)	595 (590)	641 (636)	642 (637)	644 (639)	645 (640)	646 (641)	650 (645)	660 (655)	661 (656)	662 (657)	663 (658)	668 (663)
Vaccine	**D**	**K**	**N**	**E**	**D**	**A**	**S**	**N**	**A**	**G**	**P**	**V**	**T**	**P**	**P**	**G**
B1	N	E/A	S	D	N	T	G	H	I	D	L	A	I	S	S	E
B3	N	E	S	D	N	T	G	H	T	D	L	A	I	S	S	E

Amino acid position	670 (665)	671 (666)	673 (668)	677 (672)	678 (673)	679 (674)	680 (675)	681 (676)	684 (679)	685 (680)	686 (681)	688 (683)	689 (684)	691 (686)	694 (689)	698 (693)
Vaccine	**V**	**L**	**Q**	**P**	**P**	**T**	**D**	**I**	**E**	**D**	**V**	**P**	**S**	**G**	**H**	**F**
B1	A	P	R	S/L	L	A	G	T	K	G	I	S	S/P	K	R	L/F
B3	A	P	R	S	L	A	G	T	K	G	I	S	P	K	R	L

Amino acid position	699 (694)	700 (695)	701 (696)	703 (698)	704 (699)	710 (705)	711 (706)	712 (707)	714 (709)	715 (710)	716 (711)	720 (715)	723 (518)	725 (720)	727 (722)	728 (723)
Vaccine	**P**	**S**	**R**	**S**	**T**	**G**	**L**	**M**	**S**	**G**	**T**	**G**	**S**	**R**	**M**	**T**
B1	S	G	K	N	S	D	R	M/V	T/A	S	A	E	G	H	T	A/V
B3	S	G	K	N	S	D	C	M	T	S	A	E	G	H	T	A

Amino acid position	729 (724)	733 (728)	741 (736)	750 (745)	753 (748)	774 (769)	775 (770)	788 (783)	812 (807)	830 (825)	854 (849)	858 (853)	865 (860)	869 (864)	870 (865)	900 (895)
Vaccine	**W**	**V**	**M**	**S**	**P**	**I**	**L**	**L**	**N**	**L**	**V**	**K**	**Y**	**V**	**F**	**I**
B1	R	I	I	A	S	V	F	V	D	L	A	R	H	I	L	V
B3	R	I	I	A	S	V	F	V	D	F	A	R	H	I	L	V

Amino acid position	915 (910)	920 (915)	931 (026)	939 (934)	954 (949)	957 (952)	988 (983)	1004 (999)	1005 (1000)
Vaccine	**L**	**A**	**T**	**H**	**I**	**P**	**V**	**A**	**T**
B1	S	S	A	Y	V	S	I	P	V
B3	S	S	A	Y	V	P	I	P	V

nsp3 (n=24)

Amino acid position	18	45	54	56	59	60	61	64	77	79	83	102	129	152	159	165	174	176	179	180	195
Vaccine	**D**	**R**	**T**	**P**	**S**	**I**	**S**	**T**	**V**	**A**	**V**	**A**	**A**	**I**	**T**	**M**	**I**	**S**	**C**	**A**	**I**
B1	N	Q	I	L	P/S	A	S	A	A/V	V	I	S	T	V	V	T	V	L	G	G	V
B3	N	Q	I	L	S	A	P	A	A	V	I	S	T	V	V	T	V	L	G	G	V

Amino acid position	223	235	292
Vaccine	**I**	**I**	**V**
B1	V	T	I
B3	V	T	I

nsp4 (n=23)

Amino acid position	6	9	14	29	31	32	33	34	45	58	69	72	79	83	147	152	163	164	172	180	188
Vaccine	**H**	**C**	**N**	**D**	**R**	**R**	**T**	**V**	**T**	**T**	**H**	**D**	**V**	**A**	**E**	**T**	**R**	**H**	**L**	**A**	**I**
B1	R	S	T	G	F	F	T/1	I	A	I	N	E/D	A	E	N/D	S	K/R	Y	L	T	I/V
B3	R	S	T	G	F	F	I	I	A	I	N	D	A	E	D	S	K	Y	I	T	I

Amino acid position	201	202
Vaccine	**V**	**V**
B1	A	M
B3	A	M

nsp5 (n=13)

Amino acid position	26	57	89	96	104	120	122	123	128	136	148	151	158
Vaccine	**I**	**A**	**V**	**I**	**S**	**L**	**M**	**T**	**T**	**T**	**C**	**N**	**S**
B1	V	L	I	V	P	V	V	A	I	A	H	A	A
B3	V	L	I	V	P	V	V	A	I	A	H	A	A

nsp6 (n=1)

Amino acid position	14
Vaccine	**N**
B1	S/N
B3	S

nsp7a (n=6)

Amino acid position	23	59	82	86	132	145
Vaccine	**N**	**I**	**I**	**I**	**L**	**T**
B1	D	V	T	V	P	T
B3	D	V	T	V	P	I

nsp7b (n=9)

Amino acid position	7	13	28	37	57	77	90	99	105
Vaccine	**S**	**K**	**N**	**I**	**H**	**A**	**V**	**T**	**I**
B1	G	R	K	V	Y	T	A	A	V
B3	G	R	K	V	Y	T	A	A	V

nsp8 (n=2)

Amino acid position	3	37
Vaccine	**K**	**L**
B1	R	M
B3	R	M

nsp9 (n=22)

Amino acid position	23	49	55	70	106	109	157	158	160	188	221	241	248	262	274	337	338	351	413	530	539
Vaccine	**E**	**V**	**T**	**I**	**D**	**I**	**H**	**K**	**R**	**H**	**M**	**K**	**Q**	**V**	**I**	**S**	**K**	**I**	**V**	**D**	**R**
B1	G/E	A	A	V	D/N	I/T	L	N	R	Q	M/V	T	K	I	V	T	R	V	T	E	K
B3	E	A	A	V	D	I	L	N	C	Q	M	T	K	I	V	T	R	V	T	E	K

Amino acid position	618
Vaccine	**R**
B1	Q
B3	R

nsp10 (n=16)

Amino acid position	12	16	51	57	61	66	111	191	212	217	304	353	395	396	418	428
Vaccine	**A**	**Y**	**S**	**V**	**R**	**A**	**S**	**V**	**I**	**V**	**V**	**N**	**S**	**C**	**T**	**P**
B1	A/T	H	P	I	K	T	P	I	V	T	I	S	N	R	A	P/L
B3	A	H	P	I	K	A	P	I	V	T	I	S	N	R	A	P

nsp11 (n=11)

Amino acid position	23	40	74	92	119	124	139	155	161	171	182
Vaccine	**V**	**H**	**S**	**I**	**A**	**A**	**T**	**S**	**V**	**R**	**V**
B1	A	N	P	V	T	A	T	S	I	K	V/I
B3	A	N	P	V	T	V	A	F	I	K	V

nsp12 (n=14)

Amino acid position	58	59	83	85	97	106	115	118	120	121	127	133	147	149
Vaccine	**N**	**I**	**A**	**G**	**R**	**T**	**E**	**H**	**I**	**Y**	**L**	**P**	**P**	**G**
B1	H/Y	V/I	S	N	C	I	N	L	V	H	H	L	P	E
B3	H	I	S	N	C	I	N	L	V	H	H	L	L	E

GP2 (n=35)

Amino acid position	2	5	8	13	14	19	27	29	30	39	41	42	47	48	59	74	78	83	88	95	112	114	129
Vaccine	**Q**	**Y**	**V**	**C**	**S**	**L**	**I**	**L**	**F**	**P**	**Q**	**D**	**S**	**F**	**L**	**S**	**N**	**F**	**L**	**I**	**R**	**R**	**S**
B1	R	H	V	Y	L	L/P	T	S	S	Q	P	A	F	Y	R	G	N/D	L	F	V	Q	Q	A
B3	R	H	A	Y	L	L	T	S	S	Q	P	A	F	Y	R	G	N	L	F	V	Q	Q	A

Amino acid position	138	141	184	193	194	197	199	206	224	236	246	248
Vaccine	**G**	**I**	**H**	**G**	**T**	**K**	**T**	**I**	**I**	**V**	**H**	**S**
B1	S	V	R	S	S	K/R	A	V	I/M	A/V	R	L
B3	S	V	R	S	S	K	A	V	I	V	R	L

E (n=4)

Amino acid position	3	47	54	69
Vaccine	**L**	**L**	**L**	**V**
B1	S	F	I	V/I
B3	S	F	I	V

GP3 (n=45)

Amino acid position	3	4	7	9	11	12	14	15	16	18	22	30	31	48	58	64	79	84	91	93	99	135	154
Vaccine	**H**	**Q**	**R**	**H**	**F**	**L**	**G**	**F**	**I**	**Y**	**S**	**S**	**T**	**M**	**S**	**R**	**Y**	**E**	**L**	**P**	**D**	**F**	**V**
B1	C	K	C	Y	L	F	S	I	V	H/Y	S/G	F/S	A	K	L	E	H	G/V/E	S/L	F	E	Y	I
B3	C	K	C	Y	L	F	S	I	V	Y	S	F	A	K	L	E	H	E	S	F	E	Y	I

Amino acid position	157	158	166	186	204	214	216	220	222	230	232	235	236	239	242	245	246	251	254	255
Vaccine	**G**	**H**	**A**	**L**	**V**	**I**	**R**	**P**	**L**	**T**	**I**	**D**	**L**	**S**	**R**	**K**	**F**	**R**	**V**	**V**
B1	E	R	V	F	A	T	K	P/L	V	K	N	N	I	S/F	R/H	P	V	H	A	A
B3	E	R	V	F	A	T	K	P	V	K	N	N	I	F	R	P	V	H	A	A

Amino acid position	256	257
Vaccine	**K**	**P**
B1	R	L
B3	R	L

GP4 (n=25)

Amino acid position	5	15	35	41	45	49	51	54	55	57	60	61	63	64	65	67	69	70	77	109	115	123	145
Vaccine	**T**	**I**	**E**	**A**	**M**	**D**	**N**	**R**	**P**	**G**	**A**	**A**	**E**	**E**	**I**	**F**	**K**	**S**	**V**	**H**	**G**	**W**	**H**
B1	I	L	K	G	M	K	E	Q	Y	R	T	T	K	A	S/G	L	K	P	V/I	Y	E	G	Y
B3	I	L	K	G	V	K	E	Q	Y	R	T	T	K	A	S	L	E	P	I	Y	E	G	H

Amino acid position	163	166
Vaccine	**T**	**A**
B1	S	V
B3	S	V

GP5 (n=38)

Amino acid position	2	4	6	8	15	17	20	22	41	46	56	59	60	63	71	75	79	89	90	100	104	106	111
Vaccine	**R**	**S**	**K**	**G**	**S**	**F**	**F**	**L**	**Y**	**N**	**D**	**S**	**S**	**G**	**F**	**A**	**L**	**F**	**F**	**T**	**V**	**G**	**C**
B1	K	F	R	E	F	C	L/P/F	F	R	D/N	S	S/Y	G	D/N	L	I	I	L	L	T/I	I	G/E	S
B3	K	F	R	E	F	C	L	F	Y	N	F	S	G	D	L/F	I	I	L	L	T/I	I	E	S

Amino acid position	116	119	122	123	125	126	150	154	162	171	172	173	182	195	201
Vaccine	**A**	**F**	**F**	**V**	**F**	**V**	**N**	**V**	**V**	**V**	**D**	**G**	**V**	**S**	**A**
B1	V	L	L	I	L/F	A	D	I	L	I/V	G	S	I/V	P	A/T
B3	V	L	L	I	L	A	D	I	L	I	G	S/N	V	P	A

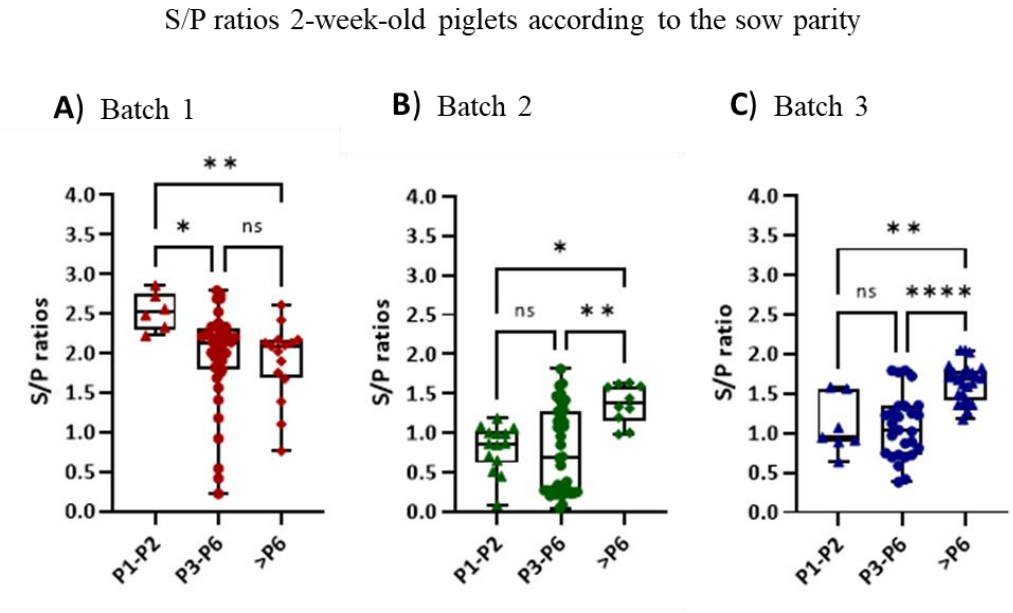
M (n=14)

Amino acid position	3	6	9	11	12	28	62	65	68	69	72	73	125	130
Vaccine	**G**	**D**	**N**	**P**	**I**	**I**	**V**	**Q**	**N**	**R**	**F**	**T**	**S**	**R**
B1	S/N	D	G	S/P	T/A	M	A	H	N/D	R	L	T/I	P	R/Q
B3	S	G	G	S	T	I	A	H	N	Q	L	T	P	R

N (n=13)

Amino acid position	13	22	32	33	35	43	48	49	51	69	100	127	128
Vaccine	**S**	**P**	**A**	**M**	**K**	**G**	**K**	**K**	**P**	**T**	**S**	**A**	**S**
B1	N	S	S	V	R	R	R	N	P/L	N	G/S	A/E	N
B3	N	S	S	V	R	R	R	N	P	N	G	A	N

Figure S10. S/P ratio values of the piglets by parity of the sow as determined by ELISA. Each dot represents an examined individual. S/P ratio values ≥0.4 are considered positive. The graphs show the S/P ratio values of the piglets at 2 weeks of age by parity of the sow in Batch 1 (A), Batch 2 (B), and Batch 3 (C).



Supplementary Animation. Transmission chains in Batch 1. The animation shows the location and movement of the animals in Batch 1. Animals with the same color correspond to a transmission chain. An asterisk symbol (*) indicates new infection cases.

3.8. REFERENCES

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Chapter 4.

Study 2

4. STUDY 2

Selection of viral variants with enhanced transmission and reduced neutralization susceptibility may explain the persistence of porcine reproductive and respiratory syndrome virus in vaccinated breeding herds.

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4.1. ABSTRACT

Objective: This study investigates the long-term evolutionary dynamics of porcine reproductive and respiratory syndrome virus (PRRSV-1) in an endemically infected and vaccinated pig herd.

Methods: Over a one year and a half period, piglets from seven farrowing batches in a 300-sow PRRSV-vaccinated farm were monitored from birth to nine weeks of age by RT-qPCR. Eighty-five PRRSV-positive samples were subjected to whole genome sequencing (Illumina Miseq), and 251 samples to ORF5 sequencing. Farm-specific PRRSV variants' impact on anti-PRRSV antibodies was evaluated using ELISA and neutralizing antibody assays. The replication kinetics and cytokine inhibition capabilities (IFN-α and TNF-α) of these variants were assessed in porcine alveolar macrophages.

Results: The study revealed fluctuating PRRSV-1 incidences in farrowing units and nurseries, attributed to two key evolutionary events: an escape variant emergence and a lateral introduction of a new strain. Initially, strain 1 variant α was swiftly replaced within weeks by variant 1β (99.5% genomic similarity), with twenty-five amino acid mutations, primarily in nsp1α, GP2, GP3, and GP5, including an additional glycosylation site and a deletion in the neutralization epitope of GP5. This shift to 1β correlated with increased incidence in nurseries and higher viral loads, with sera from 1α-exposed animals showing reduced neutralization against 1β. Consistently for in vitro assays, variant 1β demonstrated enhanced replication in porcine alveolar macrophages but no difference regarding IFN-α or TNF-α responses. Later, a new strain (strain 2, 83.3% similarity to strain 1) emerged and led to incidence resurgence because of the low cross reactivity with the previous antibodies.

Conclusion: The study highlights PRRSV's rapid adaptability and challenges in controlling its spread, underscoring the necessity for more effective vaccines and eradication approaches.

4.2. INTRODUCTION

Maintaining high health standards in modern pig production is essential for economic sustainability and ethical considerations. Porcine reproductive and respiratory syndrome (PRRS), one of the costliest and welfare-impacting diseases in pigs (Neumann et al., 2005; Holtkamp et al., 2013; Nathues et al., 2017), exemplifies these challenges.

Caused by two species, *Betaarterivirus suid 1* and *Betaarterivirus suid 2*, commonly known as PRRS virus 1 (PRRSV-1) and PRRSV-2, respectively, PRRSV is characterized by its high genetic diversity. This diversity arises from a combination of factors: high-rate substitution, frequent recombination, worldwide spread, and immune selection pressures (Forsberg, 2005; Martín-Valls et al., 2014). Such genetic and antigenic diversity facilitates infection in herds with pre-existing immunity from either prior infections, vaccinations, or both. Noteworthy, neutralizing antibodies targeting PRRSV seem to be strain-specific and have limited cross-reactivity (Martínez-Lobo et al., 2011).

Upon initial viral introduction in a naïve sow farm, a reproductive outbreak typically ensues, characterized by late-gestation abortions, premature or delayed farrowing, a rise in mummified foetuses and stillbirths, and an increase in weak-born piglets along with higher mortality rates in the farrowing units (Zimmerman et al., 2019). In cases of highly virulent strains, sow mortality is also observed (Halbur and Bush, 1997; Tian et al., 2007; Martín-Valls et al., 2023). The infection then progresses to nurseries, manifesting as respiratory disease, often exacerbated by secondary pathogens (Zimmerman et al., 2019).

A pivotal aspect of PRRS epidemiology is the vertical transmission during late gestation, resulting in the birth of viraemic piglets that can remain infectious for months and act as primary infection sources for other litters, in both the farrowing units and nurseries (Benfield et al., 2000; Rowland et al., 2003). Thus, vertical transmission is thought to be the primary contributor to the maintenance of infection within a farm (Pileri and Mateu, 2016). Consequently, most monitoring programs focus on detecting viral circulation in the farrowing units, using terms “unstable” or “stable” to indicate the virus’s presence or absence, respectively (Holtkamp et al., 2011, 2021). Monitoring is typically achieved by examining pigs at weaning or testing individual or aggregate samples, such as umbilical cords, tongue tips, and processing fluids, from piglets in the farrowing units (Martín-Valls et al., 2018; Vilalta et al., 2018; Baliellas et al., 2021).

In the absence of intervention, PRRS often becomes endemic, with periodic reproductive and respiratory disease rebounds (Pileri and Mateu, 2016). Given the widespread nature of the virus, novel introductions of different PRRSV isolates, referred to as "lateral introductions", are common. The consequences of such introductions vary widely, ranging from subclinical infections to severe outbreaks, and are influenced by the strain's virulence and the existing immunity's cross-reactivity.

Control of PRRSV requires a multifaceted approach that encompass vaccination, biosecurity, animal movement management, as well as accurate monitoring and diagnostics. Vaccination of sows is a common practice, starting with gilts and followed by recall vaccinations every few months. Live attenuated vaccines are typically employed due to limited efficacy of the inactivated ones (Zuckermann et al., 2007).

However, even with robust vaccination and biosecurity protocols, PRRSV can persist within herds. Unfortunately, there is limited understanding of the specific mechanisms allowing the virus to persist within these populations, hindering the development of alternative strategies beyond resorting to herd depopulation or closure, both of which carry substantial economic implications (Torremorell et al., 2003; Corzo et al., 2010).

This study aims to elucidate long-term evolutionary patterns of PRRSV in farms where the virus persists with conventional control measures. We closely monitored an endemically infected farm for over one year and a half, tracking piglets from birth to the end of their nursery period. Our focus was on unravelling the temporal evolution of the virus and correlating it with the epidemiological impacts, humoral responses, and potential mechanisms of immune evasion. Insights from this study would be crucial for developing more effective control strategies in farms grappling with endemic PRRSV infections.

4.3. MATERIALS AND METHODS

4.3.1. Study farm and follow-up chronology.

The study farm housed a total of 300 breeding sows, which were managed using a three-week batch farrowing system. Piglets were weaned at four weeks of age and then raised in nurseries until nine weeks of age before transferring to a fattening unit to reach market weight (100 kg). Before the study commenced, the farm had a history of routine PRRSV

positivity (for years) in weaned pigs as identified by RT-qPCR. Replacement gilts, purchased from a PRRSV-negative herd at six months of age, were quarantined until their first service and received at least two doses of a modified live PRRSV vaccine (Porcilis® PRRS, MSD, Spain) before their first gestation. A blanket vaccination protocol was implemented for all sows and gilts, involving at least three vaccinations annually.

Figure 1 depicts the sampling scheme employed in this study. The initial farm sampling (Batch 0) was followed by six subsequent batches (Batches 1 to 6, from May 2018 to July 2019) to monitor PRRSV status. Each batch involved monitoring at least ninety-five piglets, from sows of various parities, from birth to nine weeks of age (about forty-one farrowings per batch, averaging 500 piglets in total). This sample size allowed to detect a ≥3% infection rate with 95% confidence. Umbilical cords (UC) were collected at birth, and blood samples were obtained at various time points: once or twice in the farrowing unit (at three or four weeks of age for batches 2, 4, 6, and at two and four weeks of age for batches 1, 3, 5) and twice in the nurseries (at six and nine weeks of age). Each animal was ear-tagged for identification. An additional sampling (Batch 7, September 2020) was conducted fourteen months after Batch 6 sampling (thirty-two months after Batch 0 sampling), with umbilical cords collected at birth and blood samples collected from three-, six-, and nine-week-old animals. The farm's productivity data and mortality rates were recorded throughout the study period.

The animal study was reviewed and approved by Ethics Committee in Animal and Human Research of the Universitat Autònoma de Barcelona (approval numbers 3221-CEEA-UAB and CEEAH-5691 for the project and the procedure, respectively).

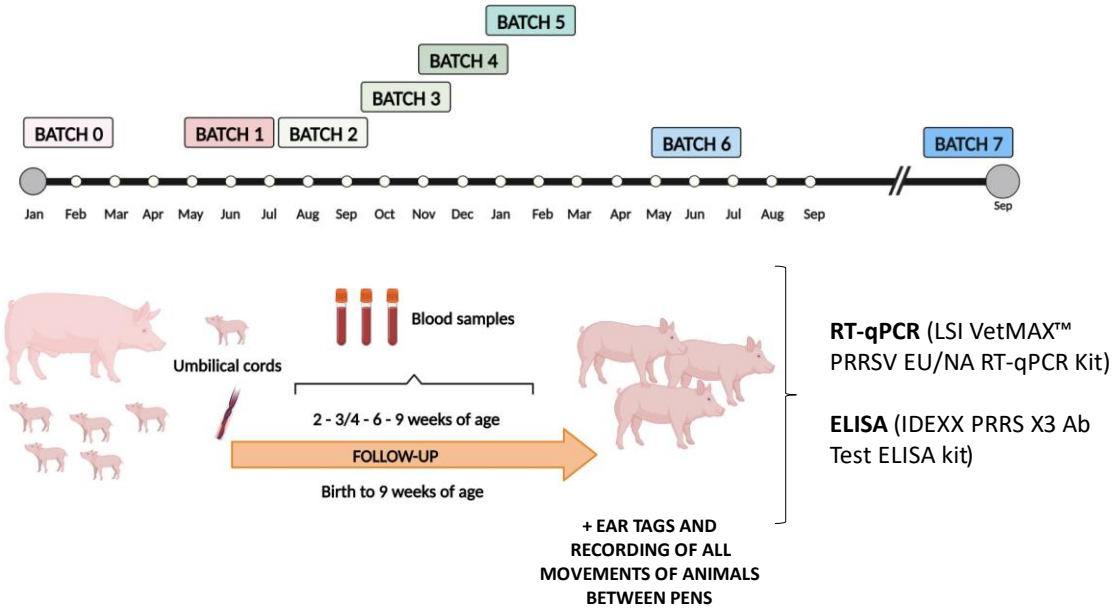

Figure 1. Graphical depiction of the sampling scheme in the farm. Seven batches of piglets (numbered #0 to #6) were followed periodically from birth to nine weeks of age to assess the dynamics of PRRSV infection in the farm. Umbilical cords were collected at birth followed by periodic blood sampling. Samples were analysed by RT-qPCR and ELISA to assess the infection status. An additional sampling (Batch 7) was conducted fourteen months after Batch 6. Created with BioRender.com.

4.3.2. PRRSV detection.

Upon arrival at the laboratory, the UC and blood samples were processed as previously described (Martín-Valls et al., 2018; Clilverd et al., 2023). Briefly, UC were minced into 5 ml of minimal essential medium, centrifuged at 12,000 x g for 5 minutes, and the supernatant was aliquoted and stored at -80°C. Blood samples were centrifuged at 300 x g for 5 minutes and subsequent serum aliquots were preserved at -80°C.

Viral RNA extraction was performed using NucleoSpin® RNA virus kit (Macherey-Nagel, Germany) with a final elution volume of 50 µl. Each extraction included a PRRSV positive sample and DEPC water as controls. PRRSV RNA detection was performed with a commercial RT-qPCR kit designed to detect both PRRSV-1 and PRRSV-2 (LSI VetMAX™ PRRSV EU/NA kit, Thermo Fisher Scientific) with an internal positive control included in each sample. In order to increase the specificity of the test, samples yielding C_t values <37 were considered positive, while those with C_t values between 37.1 to 39.9 were deemed suspicious. Suspicious samples, upon retesting, were considered negative if C_t values remained ≥37.1.

Vertical transmission events (VTE) were identified based on the detection of at least one positive UC per litter using RT-qPCR. Incidence rates were calculated by dividing new cases by the number of susceptible animals during each observation period. If an animal tested positive in two consecutive samplings, it was categorized as a new case only at the first positive sampling time. Moreover, to account for infected animals that could have been undetected, pigs seropositive at nine weeks of age without prior RT-qPCR detection were also considered new cases. Animals with missing data were excluded from the calculations.

In addition, tonsils from approximately 10% of the sows (28 animals) were tested for PRRSV using RT-qPCR. Sampling was done at the slaughterhouse. Tonsil homogenates were subject to RNA extraction with TRIzol reagent and RT-qPCR analysis as described above.

4.3.3. PRRSV isolation.

Porcine alveolar macrophages (PAM), sourced from high-health-status pigs via bronchoalveolar lavage (Mayer and Lam, 1984), were used for PRRSV isolation from RT-qPCR-positive samples. Prior to usage, PAM were tested negative for PRRSV, porcine circovirus 2, and mycoplasma by RT-qPCR. In each followed pig batch, viral isolation was attempted only from samples (all UC and at least 20% of the blood samples) with C_t values ≤32.0. The isolation was restricted to a single passage to prevent any potential bias in the results of the whole genome sequencing (Cortey et al., 2018). On observing cytopathic effect, cell culture supernatants were collected, centrifuged at 400 x g for 10 minutes, aliquoted, and preserved at -80°C until use.

4.3.4. Sequencing.

To gain a comprehensive understanding of the viral genetic variability and evolutionary patterns, at least 20% of the positive samples from every batch were randomly selected for next generation sequencing (NGS) analysis. Viral RNA was extracted from the isolates using the TRIzol™ reagent with a 20 µl elution volume. Samples were then sequenced using Illumina Miseq technology, following a previously

described protocol (Clilverd et al., 2023) with no prior amplification steps involved. A total of eighty-five PRRSV isolates were subjected to whole genome sequencing. For a selection of sequences, the nucleotide frequency per position was determined and the proportion of different amino acids within the quasispecies was inferred.

All RT-qPCR-positive samples with a C_t value ≤32.0 underwent amplification and Sanger sequencing of ORF5 (n= 251), which encodes for the virus major envelope protein and has conventionally been employed for phylogenetic analysis of PRRSV. A previously described protocol (Mateu et al., 2003) and tailor-made oligonucleotide reverse primers were used (Supplementary Table 1).

The consensus sequences obtained from both whole genome and the ORF5 sequencing were submitted to GenBank with the Accession Numbers OR667160 to OR667244, and OR620633 to OR620890, respectively.

4.3.5. Phylogenetic and evolutionary analysis.

To elucidate the genetic relationships and diversity of the obtained PRRSV sequences in this study, a phylogenetic analysis was conducted that encompassed the whole genome and ORF5 sequences. For phylogenetic tree construction, Bayesian inferences through MrBayes (available at https://ngphylogeny.fr) with one million iterations was employed. To quantify genetic variations, p-distances were calculated using MEGA X (Kumar et al., 2018), enabling the assessment of inter- and intra-batch and viral variant diversity for both the whole genome and ORF5 sequences. In addition, the potential occurrence of recombination events in the whole genome sequences were investigated employing GARD algorithm method (Pond et al., 2006) and RDP5.0 software (Martin et al., 2021). Furthermore, a comparative analysis of the predicted protein sequences' amino acid composition was conducted, enabling a detailed examination of differences and similarities between the viral strains and variants.

A collection of complete genomes and ORF5 sequences of PRRSV-1 strains, which included the five PRRSV vaccines that have been commercially licensed in Spain, were retrieved from GenBank (Supplementary Table 2). To conduct a comparative analysis, phylogenetic trees were constructed using MrBayes and genetic distances were quantified (p-distance) as abovementioned.

Prediction of N-glycosylations in the viral structural glycoproteins was carried out using Net-N-Glyc (Gupta and Brunak, 2002) available at https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/.

4.3.6. Adaptation to grow on MARC-145 cells and viral production in PAM.

Viruses successfully isolated from serum samples were subjected to adaption in MARC-145 cells. Confluent monolayers of MARC-145 cells in 24-well plates were inoculated with RT-qPCR positive samples. Representative isolates of each variant (1α, 1β, and 2) were adapted, with the fifth passage designated as the working stock. Titration of the viral stocks was performed by the $TCID_{50}$ method, following the Reed and Muench (1938) calculation. To identify mutations associated with the virus's adaptation to MARC-145 cells, whole genome sequencing was performed as described in section 2.4. The consensus sequences of variants 1α, 1β, and 2 were submitted to GenBank, corresponding to the Accession Numbers OQ440239, OQ440240, and OQ440241, respectively. Supplementary Table 3 shows the differences between the PAM-isolated and the MARC-145 adapted isolates.

The original serum samples used for isolation and adaptation were also used to propagate the same variants in PAM. The fifth passage of 1α, 1β, and 2 in PAM was used as the working stock for further experiments.

4.3.7. Serological analyses (ELISA and viral neutralization test).

All serum samples collected in the farrowing units and from nine-week-old animals were analysed by ELISA to determine the presence of anti-PRRSV antibodies (IDEXX PRRS X3 Ab Test, IDEXX, United States). Furthermore, over 60% of six-week-old animals in each batch were randomly selected for the same analysis. The ELISA results were expressed as sample to positive control (S/P) optical density ratios according to the manufacturer's instructions. S/P ratios ≥0.40 were considered positive.

The presence of neutralizing antibodies was assessed against the three viral variants identified in this study (1α, 1β, and 2) and the vaccine strain employed on the farm. For this purpose, a subset of serum samples from two-week-old animals in batches 1, 5, 6, and

7 was chosen to examine maternally-derived antibody titres. These particular batches were selected as only one viral variant was circulating in their respective farrowing units at that time. Titres of neutralizing antibodies of those samples were assessed against the circulating viral variant in the corresponding batch. For batches 1 and 5, sera were also tested against the subsequently appearing variant or strain. In addition, serum samples from fourteen sows in Batch 1 (collected two weeks after farrowing) were tested against the circulating viral variant in that batch and the emerging one in Batch 2. All samples were further tested against the vaccine virus used in the farm, serving as a reference for comparison.

To determine antibodies in piglets after the infection by a given viral variant, nine-week-old animals that had been infected before six weeks of age were selected, ensuring sufficient time for the development of neutralizing antibodies. In this case, the assessment of neutralizing antibodies was performed against the three viral variants but not the vaccine, as piglets were not vaccinated.

The viral neutralization test (VNT) was conducted using a previously described protocol (Yoon et al., 1994) with minor modifications. Samples were tested in duplicate, and the titre of a sample was calculated as the average of two replicas. Samples with replicas that differed more than one dilution were retested. Neutralizing antibody titres were expressed as $\log_2$ values with titres ≥ 3 $\log_2$ considered positive. To confirm neutralization, cell cultures were stained with anti-PRRSV-1 nucleocapsid protein (N) antibody (clone 1C5H; Ingenasa, Madrid, Spain) followed by a secondary fluorescein-labelled goat anti-mouse IgG2b (H + L) (Jackson ImmunoResearch, Spain).

4.3.8. Viral replication kinetics.

The replication kinetics of viral variants 1α and 1β were evaluated in PAM. Briefly, PAM were seeded overnight in 96-well plates at a density of 1.2 x 105 cells/well. Then, they were inoculated with each variant at a multiplicity of infection (MOI) of 0.1. After 90 minutes incubation, unbound virus was washed away and the cultures were replenished with fresh culture medium, being incubated for different times (0, 12-, 24-, 48-, and 72-hours). Supernatants were collected to assess the extracellular virus, while cells were fixed in 150 µL of methanol-ethanol (75:25) at -20 °C for further analysis.

RNA from the supernatants was extracted using MagMax Core Nucleic Acid Purification Kit (Applied Biosystems, Thermo Fisher Scientific, United States), following the manufacturer's instructions. The quantification of viral loads was performed using a commercial RT-qPCR kit, as described in section 2.2. A standard curve correlating C_t values to viral loads was established using a decimal dilution series of each viral isolate, tested in triplicates.

Furthermore, the proportion of infected PAM at each timepoint was determined using the fixed PAM cultures. For this purpose, cells were labelled with mAb 1CH5 along with a secondary fluorescein-labelled goat anti-mouse IgG2b as for the neutralization test (section 2.7). Nuclear staining was performed with ProlongTM Gold Antifade Mountant with DAPI stain (Invitrogen, Thermofisher Scientific, United States). PRRSV-positive cells were counted in five fields per replicate (400x magnification) with at least 100 cells per field, using an inverted fluorescence microscope (Optika® Italy IM-3FL, Optika S.r.l, Italy).

4.3.9. Attachment of different viral variants to PAM.

To explore factors influencing the replication kinetics of viral variants, the assessment of the attachment to PAM was conducted. PAM were seeded overnight, then detached and transferred to 1.5 mL tubes (65,000 cells/tube), where cells were inoculated with each variant at an MOI of 1 on ice for 60 minutes. Unbound virus was washed away, and cells were fixed with cold methanol-ethanol (75:25) at -20ºC for a minimum of 20 minutes. Three replicates were prepared for each variant, and mock-inoculated cells were used as the negative control.

Immunofluorescence staining along with NucBlue nuclear staining was performed. PRRSV-1 N was labelled with primary antibody 1CH5 (Ingenasa, Spain), followed by a secondary antibody anti-mouse IgG2b conjugated to Alexa Fluor 647 (Invitrogen, Thermo Fisher Scientific, United States). Negative controls included time zero samples, mock-inoculated cultures, and irrelevant mouse IgG2b isotype-matched antibody staining (Bio-Rad, UK). After staining, slides were mounted with Prolong Glass Antifade Mountant with NucBlue (Invitrogen, Thermo Fisher Scientific, United States). Confocal analysis was

performed using a Leica TCS SP5 confocal microscopy. Channel merging and image processing were conducted using Fiji software (Schindelin et al., 2012).

4.3.10. Inhibition of IFN-α and TNF-α.

To determine whether differences in viral replication among different viral variants could be attributed to their differential impact on antiviral cytokines, IFN-α and TNF-α were examined. PAM were overnight seeded in 96-well plates (1x105 cells/well) followed by stimulation with different viral variants at MOI 0.5, 10 µg/mL poly I:C (InvivoGen, United States), or culture medium, in various combinations: i) virus only, ii) poly I:C only, iii) virus and poly I:C simultaneously, iv) virus followed by poly I:C 6 hours after the inoculation of the virus, v) poly I:C followed by the virus 6 hours later, and vi) culture medium. After 24 hours of stimulation, cell culture supernatants were collected and examined by ELISA to measure IFN-α and TNF-α levels (Porcine IFN-α ELISA kit and TNF α Porcine ELISA kit, Invitrogen, Thermo Fischer Scientific, United States). The optical densities from mock-inoculated cultures were used to evaluate the background of the assay and was subtracted from the obtained values with each stimulus.

4.3.11. Statistical analyses.

Statistical analyses were performed using GraphPad Prism v.10. Significance was set to $p<0.05$. Comparison between incidences of PRRSV infection were performed using the $\chi 2$ (Fisher exact test when needed). Comparison of average S/P ratios or neutralization titres were performed using the Kruskal-Wallis test. Comparison of the proportions of infected cells in the examined replicas were calculated using a Mann-Whitney test. Linear regression analysis was used to correlate incidences between different production phases.

4.4. RESULTS

4.4.1. PRRSV infection dynamics in the farm and vertical transmission events.

During the study, a follow-up of 685 piglets from 157 litters was conducted, spanning seven consecutive batches over a period of approximately one and a half years (Batches 0 to 6). The data regarding the sows and litters followed in each batch has been summarized in Table 1. Interestingly, marked variations were observed between the proportions of PRRSV-positive litters at birth, and the incidences recorded in the farrowing units and nurseries (Table 1). It is worth noting that increased proportions of positive litters at birth could not be significantly correlated with increased transmission rates in the farrowing units. Similarly, the incidence in the farrowing units did not correlate with an increased incidence in nurseries, and vice versa.

Table 1. Basic data on the followed pig batches with indication of the numbers of litters and pigs, parity ranges for the sows, incidence of PRRSV infection in the farrowing units and nurseries, and the viral variant/strain detected at each timepoint.

Batch	Nº litters examined at birth/followed	Range of sows' parities	Pigs followed (1-9 woa¶)	Litters with PRRSV+ piglets at birth	Incidence in farrowing units	Incidence in nurseries$	Viral variant/strain present
0	9/9	1-8	72	3 (33.3%) ($CI_{95\%}$: 9.0-69.1%)	1.5%	60.6%	1α
1	32/25	1-8	113	6 (18.8%) ($CI_{95\%}$: 7.9-37.0%)	13.3%*	36.5%***	1α
2	27/24	2-9	98	3 (11.1%) ($CI_{95\%}$: 2.9-30.3%)	3.2%*	86.7%***	1β
3	27/18	1-7	97	4 (14.8%) ($CI_{95\%}$: 4.9-34.6%)	3.1%	56.7%***	1β
4	34/34	1-7	115	0 (0.0%) * ($CI_{95\%}$: 0.0-12.6%)	0.9%*	15.9%***	1β/2
5	28/27	1-10	95	3 (10.1%) † ($CI_{95\%}$: 2.8-29.4%)	11.8%**	92.6%***	2
6	18/12	1-5	95	0 (0.0%) ($CI_{95\%}$: 0.0-21.9%)	1.1%**	65.9%***	2
7[a]	5/0	1 + 6/7	N.A.	1 (20%) ($CI_{95\%}$: 1.05%-70.12)	46%	62.5%	2
Totals/ Averages	**175/146**	**1-10**	**685**	**10.9%**	**5.0%±5.3%**	**59.3%±26.8%**	**N.A.**

a= In this case a cross-sectional sampling was performed. Data represent prevalence not incidence. $= Differences were calculated with regards to the previous batch. N.A.= Does not apply.
¶ woa= weeks of age. † $p<0.10$; *$p<0.05$; **$p<0.01$; ***$p<0.001$

RT-qPCR allowed to compare the viral loads among animals in each batch. Upon comparing the C_t values of pigs that were infected in the nurseries within each batch, the results showed significant differences (Figure 2). Specifically, with each rise in nursery incidence, there was a corresponding decrease in the average C_t values of viraemic animals detected in those nurseries (R2= 0.853; p=0.001), thereby implying that increased incidences were correlated with increased individual viral loads (Figure 2).

Regarding the examination of sow tonsils for the presence of PRRSV, all but one were negative by RT-qPCR. The positive sow (Ct=37) could not be related with a VTE.

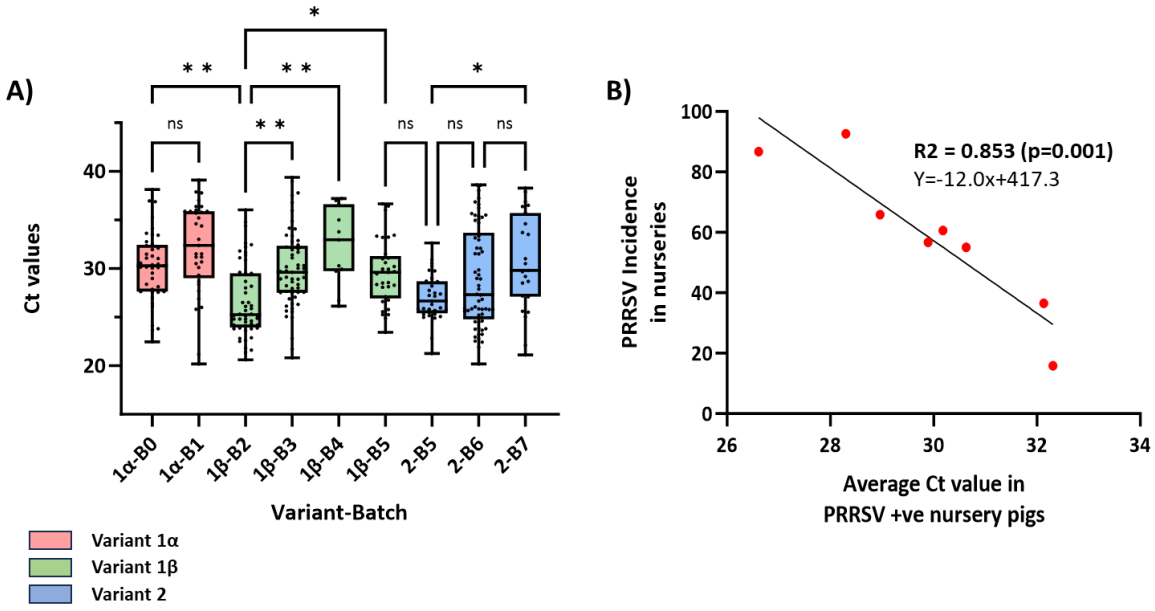

Figure 2. Average C_t values per batch of piglets infected in the nurseries (A) and correlation of the C_t values with the incidence of the infection in each nursery (B). A) The graph compares C_t values of RT-qPCR positive animals in batches 0 to 7 (B1-B7). Each dot represents an examined individual. The box and whiskers plot shows the minimum, maximum, median, and quartiles 25 and 75. The X-axis shows values for each viral variant/strain. Boxes with the same colour represent the same viral variant/strain. ns= not significant. B) The graph shows the correlation between the average C_t values of each batch (red dots) with the cumulative incidence in the nursery in that batch.

4.4.2. Phylogenetic and recombination analyses and viral variant characterization.

Figure 3 displays a Bayesian phylogenetic tree that was constructed using the whole genome sequences obtained during the study. This analysis initially identified a PRRSV-1 strain circulating from Batch 0 to 5. This particular strain presented distinct variants: 1α, which was detected as the solely variant in Batch 0 and 1, and 1β, which was present from Batch 2 to 5. Both variants shared >99.5% of nucleotide similarity (Supplementary Table

4) but were distinguishable by certain marker mutations and deletions. Subsequently, during the nursery period in Batch 5, a new strain (referred to as strain 2) was identified in some nursery samples, but not in the farrowing units. This strain 2 showed only 83% nucleotide similarity to the previously observed viral variants 1α and 1β (Supplementary Table 4), and eventually became the dominant strain, persisting as the sole strain detected until the end of the study (Batch 7). The analysis of ORF5 sequences from all RT-qPCR positive samples with C_t values <32 (Supplementary Figure 1) confirmed the absence of other variants or strains. Furthermore, when comparing with sequences from commercially available attenuated vaccines, the variants found in our study did not correspond to the vaccine used on the farm, nor to any other available vaccine (Supplementary Figure 2, Supplementary Figure 3, and Supplementary Table 5).

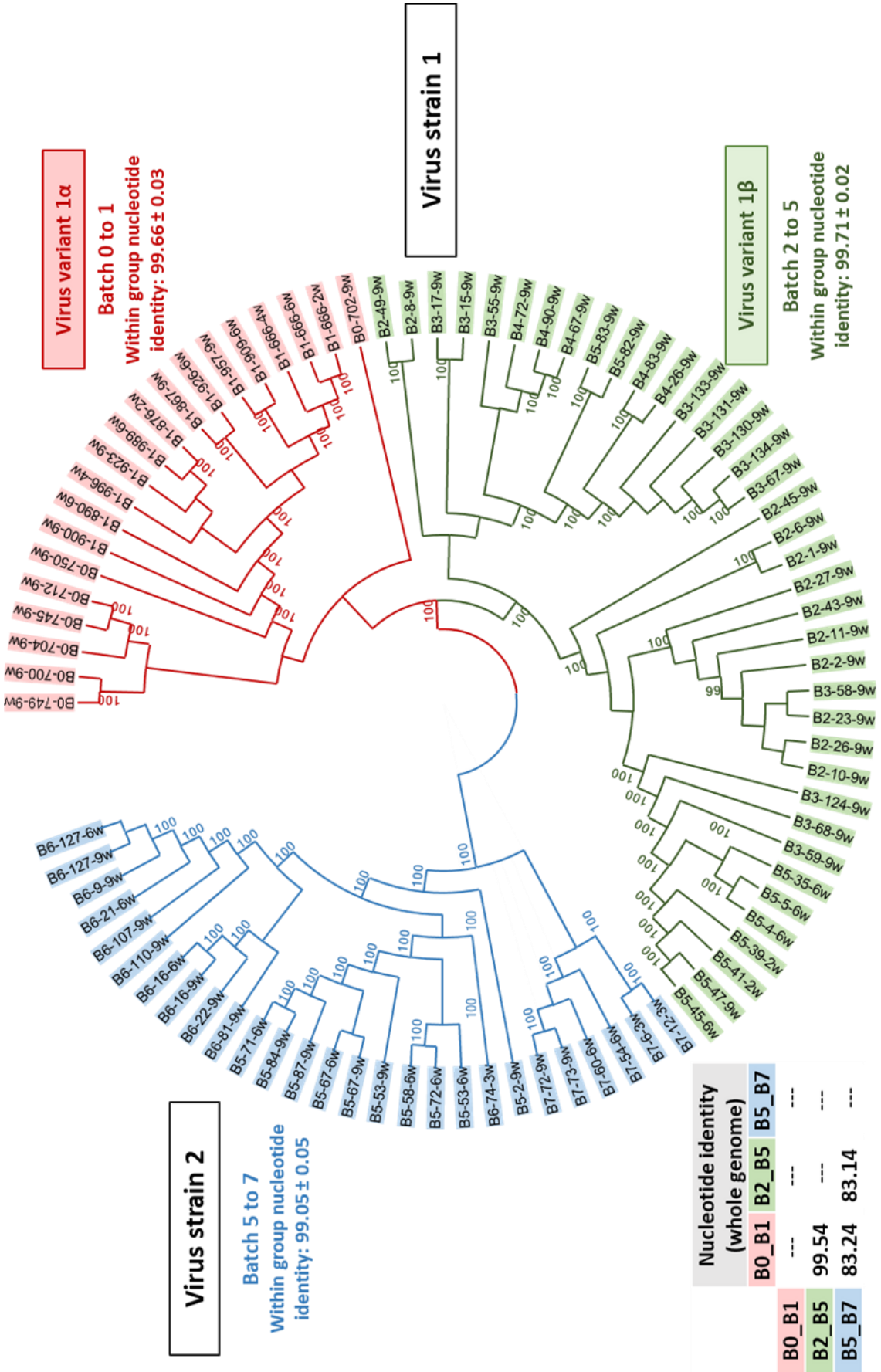
Virus variant 1α
Batch 0 to 1
Within group nucleotide identity: 99.66 ± 0.03
Virus strain 1
Virus variant 1β
Batch 2 to 5
Within group nucleotide identity: 99.71 ± 0.02
Virus strain 2
Batch 5 to 7
Within group nucleotide identity: 99.05 ± 0.05
Nucleotide identity (whole genome)
B0_B1 B2_B5 B5_B7
B0_B1 --- --- ---
B2_B5 99.54 --- ---
B5_B7 83.24 83.14 ---

Figure 3. Bayesian tree showing the phylogenetic grouping of the whole genome sequences obtained in this study. Posterior probabilities higher than 70% are shown. All isolates from batches 0 and 1 belonged to the 1α cluster (red). All isolates of batches 2 to 4 and most of the Batch 5 sequences belonged to variant 1β (green). In Batch 5, a new strain appeared in the nurseries and subsequently in batches 6 and 7 it became the only detectable PRRSV strain (strain 2, in blue). No statistically significant variants were found for strain 2.

The comparison of amino acid compositions of viral proteins between variants 1α and 1β, inferred from their consensus nucleotide sequences, revealed that they differed by only twenty-five positions across the whole genome for all examined isolates (Figure 4 and Supplementary Table 6). Of note, these mutations were not randomly distributed. For example, nsp1α (180 aa), comprising only 3.8% of the genome's coding regions, accumulated five non-synonymous mutations, representing 20% of the total number of non-synonymous mutations in the entire genome. Similarly, GP2 (249 aa, 5.3% of the coding regions) and GP3 (263 aa, 5.5% of the coding regions) exhibited four and three non-synonymous mutations respectively, with GP3 also gaining an additional glycosylation site at position 100. In contrast, nsp2 of variant 1β (17.8% of the coding regions of the genome and the largest protein in PRRSV) only harboured three non-synonymous mutations (12% of the total number of this type of mutations) compared to 1α. To note, variant 1β had a deletion at position 58 of GP5, downstream of the neutralization epitope in this protein, and the fixation of a glycosylation site at position 46, which was also seen in some isolates of 1α. These findings suggest that the selection pressures on different viral proteins were different.

Interestingly, both variants 1α and 1β presented two deletions in GP3, at positions 237 and 244 (compared to Lelystad virus), and a double deletion in GP4, at positions 55 and 65, which correspond to the neutralization epitope described by Costers et al. (2010a).

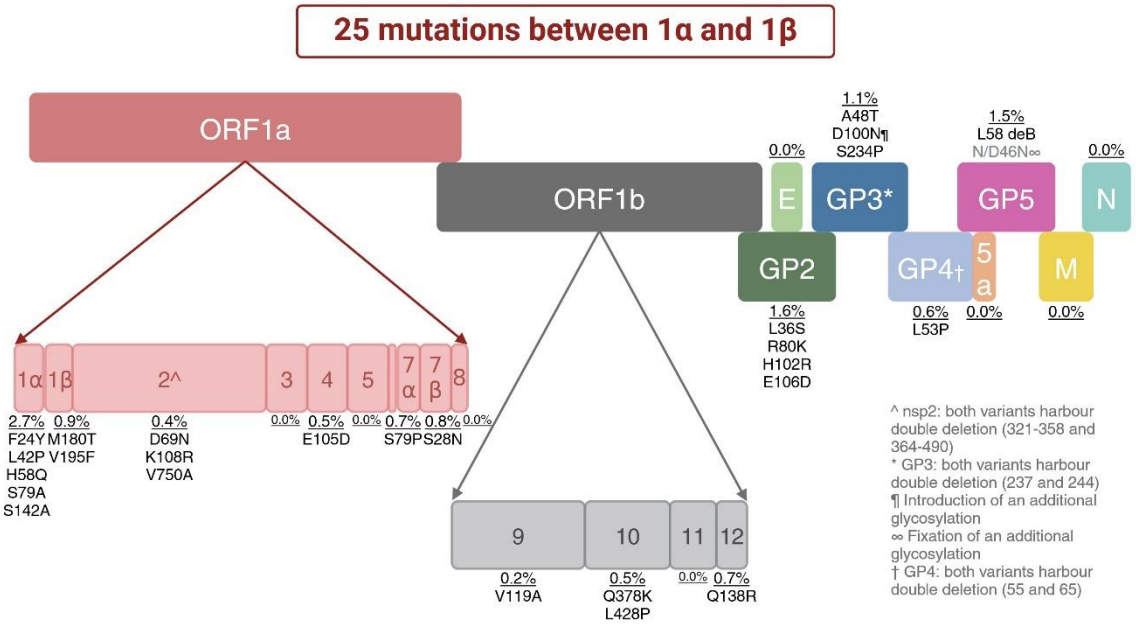

Figure 4. Distribution of non-synonymous mutations and deletions in variant 1β compared to variant 1α. The figure shows the non-synonymous mutations across the viral genome in all sequenced isolates for variant 1β (synapomorphic traits) that were absent in the consensus sequences of 1α, with indication of the protein affected and, for ORF1a and ORF1b, the non-structural proteins (nsp) involved. Amino acids are represented with a single-letter code. The first letter indicates the predominating amino acid in variant 1α, the number the position in the protein, and the second letter indicates the amino acid in variant 1β. Created with BioRender.com.

Given the rapid replacement of variant 1α by variant 1β and the presence of relatively high-fixed mutations (25), we examined whether different mutations or groups of mutations co-occurred or independently appeared in different animals. Analysis of viral quasispecies in 1α isolates for nsp1 and GP2 revealed the existence of all fixed mutations in 1β, although the majority were at low frequencies (<5% of reads) (Table 2). However, high frequencies of F24Y and L42P in nsp1α seemed to be linked one to another in some animals but not to other mutations. Notably, higher mutation frequencies in nsp1α were not related to increased frequency of the mutations in GP2 or the new glycosylation in GP5. These findings suggest that the selection of mutations in different proteins of the virus probably did not occur in a single animal, and the generation of the predominant set of β mutations was not the outcome of gradual selection from a single variant.

Table 2. Frequency of mutations fixed in variant 1β within the quasispecies of variant 1α (n= 20 animals). The table shows the frequency, expressed over 1, of the indicated mutations for nsp1α and GP2. The last column shows the frequency of an additional glycosylation in GP5 that was already preeminent in some 1α isolates as a reference. Shadowed in yellow frequencies ≥0.2; in orange between 0.1 and 0.19; in blue 0.9-0.05; not shadowed <0.05.

Batch - Animal	Viral protein	nsp1α					GP2				GP5
Mutation	F24Y	L42P	H58Q	S79A	S142A	L36S	R80K	H102R	E106D	N/D46N
B0-700-8w	0.13	0.16	0.06	0.03	0.05	0.04	0.03	0.04	0.01	0.98
B0-702-8w	0.09	0.1	0.95	0.02	0.06	0.04	0.04	0.04	0.01	0.06
B0-704-8w	0.1	0.16	0.04	0.05	0.03	0.05	0.04	0.02	0.01	0.05
B0-712-8w	0.12	0.16	0.04	0.04	0.05	0.03	0.03	0.02	0.01	0.03
B0-745-8w	0.14	0.15	0.04	0.04	0.05	0.03	0.02	0.02	0.01	0.03
B0-749-8w	0	0	0	0	0.02	0.01	0	0.01	0	0.91
B0-750-8w	0.06	0.1	0.03	0.03	0.03	0.03	0.03	0.04	0.02	0.02
B1-666-2w	0.01	0.04	0.02	0.01	0.01	0.05	0.03	0.01	0.01	1
B1-666-4w	0.01	0.07	0.04	0.04	0.02	0.02	0.02	0.02	0.02	1
B1-666-6w	0.04	0.01	0.01	0.01	0.01	0.05	0.02	0.02	0.01	1
B1-867-9w	0.01	0.06	0.02	0.05	0.02	0.02	0.02	0.02	0.02	1
B1-876-2w	0.02	0.07	0.03	0.03	0.03	0.04	0.03	0.04	0.03	0.02
B1-890-6w	0.02	1	0.02	0.03	0.02	0.03	0.04	0.02	0.01	0.05
B1-900-9w	0.03	0.06	0.02	0.02	0.03	0.02	0.01	0.01	0	0.02
B1-909-6w	0.05	0.04	0.03	0.02	0	0.02	0.02	0.02	0.01	0.02
B1-923-9w	0.04	0.05	0.02	0.04	0.04	0.06	0.05	0.04	0.01	0.06
B1-926-6w	0	0	0	0	0	0.01	0	0	0	0.04
B1-957-9w	0.03	0.05	0.02	0.02	0.01	0.01	0.02	0.03	0.02	0.02
B1-989-6w	0	0	0.01	0.01	0	0.02	0	0.01	0	0
B1-996-4w	0.03	0.07	0.05	0.03	0.03	0.06	0.06	0.08	0.05	0.98

Next, a recombination analysis was conducted to determine if recombination could be detected in the obtained sequences. The results (Supplementary Figure 4) showed that recombination did occur between variants 1α and 1β and affected a segment of 129 nucleotides in a highly variable region of the nsp2 (positions 2,235-2,958 according to the alignment with PRRSV-1 prototype Lelystad; Accession number NC_043487). This suggests that both variants co-existed within the farm for some time and co-infected some individuals.

4.4.3. Serological analyses.

The presence of maternally-derived antibodies was determined at two and four weeks of age by ELISA (Figure 5). The results showed that significant differences can be observed for two-week-old-piglets between batches where the same variant/strain was circulating. Differences were not observed at four weeks of age. Moreover, S/P values were not correlated with VTE frequencies nor with the incidence in the farrowing units (data not shown). Supplementary Figure 5 shows the S/P values for piglets of six and nine weeks of age.

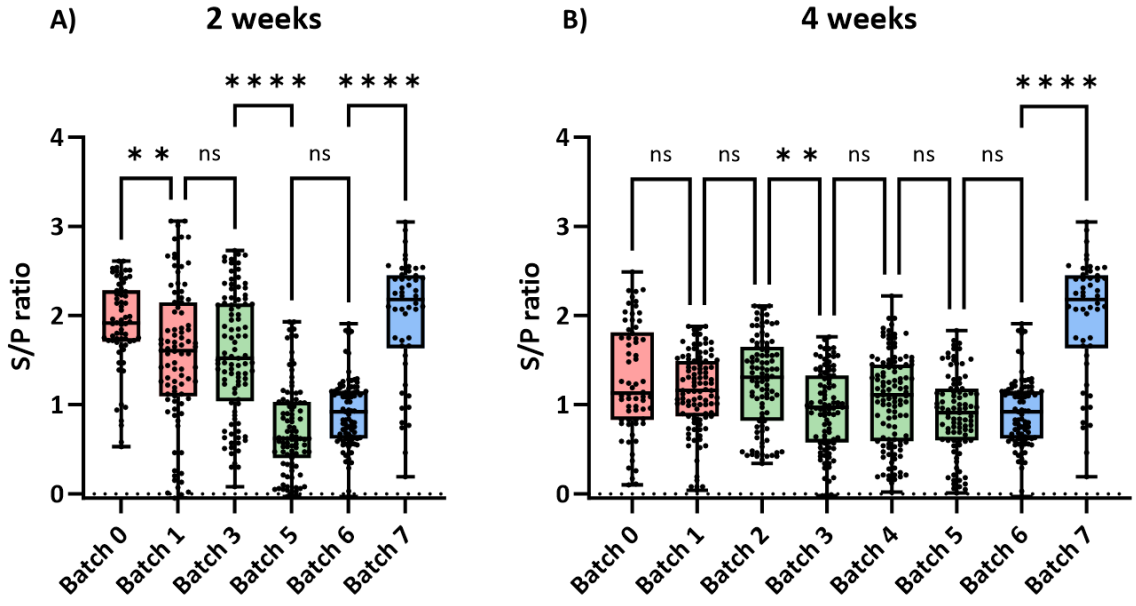

Figure 5. Antibody levels against the viral nucleocapsid protein expressed as S/P ratios as determined in ELISA. Each dot represents an individual. A) Animals examined at two weeks of age (batches 2 and 4 were not examined at that age); B) Animals examined at four weeks of age. ns=non-significant differences. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Since sera from sows and piglets present in Batch 1 were available, namely preceding the emergence of variant 1β, it was possible to test their capacity for neutralizing 1β (Figure 6). In ten sows (71%), the titres against 1α were equal or higher than the titres obtained for 1β neutralization, with differences ranging from 1 to 5 log_2. Remarkably, the sera of six sows were completely devoid of any neutralizing capacity against 1β. Similarly, when two-week-old piglets were analysed, most of them (24/31; 77.4%) showed higher titres against 1α compared to 1β, with differences ranging from 0.5 to 3.5 log_2.

Interestingly, within the group of fourteen sows, four had transmitted vertically the infection to their offspring. Of these, three had neutralization titres against 1α (4.6-7.0 log_2) and the fourth sow was negative against 1α or 1β despite having a titre >5 log_2 against the vaccine strain (data not shown).

Vaccine virus was used as a reference in the VNT. The results showed that titres against vaccine virus and 1α in both sows and piglets were not significantly different but were higher than the titres against 1β ($p<0.05$). Levels of neutralizing antibodies against the vaccine in the sows had limited predictive value for the results against 1α ($R2= 0.3897$, $p=0.017$) and even less against 1β ($R2= 0.2752$, $p=0.054$) (Supplementary Figure 6).

In Batches 5, 6, and 7, two-week-old pigs were assessed for levels of neutralizing antibodies. All animals analysed after Batch 5 had no detectable antibodies against 1α or 1β, indicating that memory of those infections had faded out from the farm (data not shown).

When nine-week-old piglets were examined in the VNT, only animals from Batch 1 (6/15; 40%) had titres ≥2log_2 against variant 1α, the one circulating in that batch (range 2-8 log_2). No neutralizing capacity was detected against any of the examined viruses in nine-week-old pigs from batches 2-7 (data not shown).

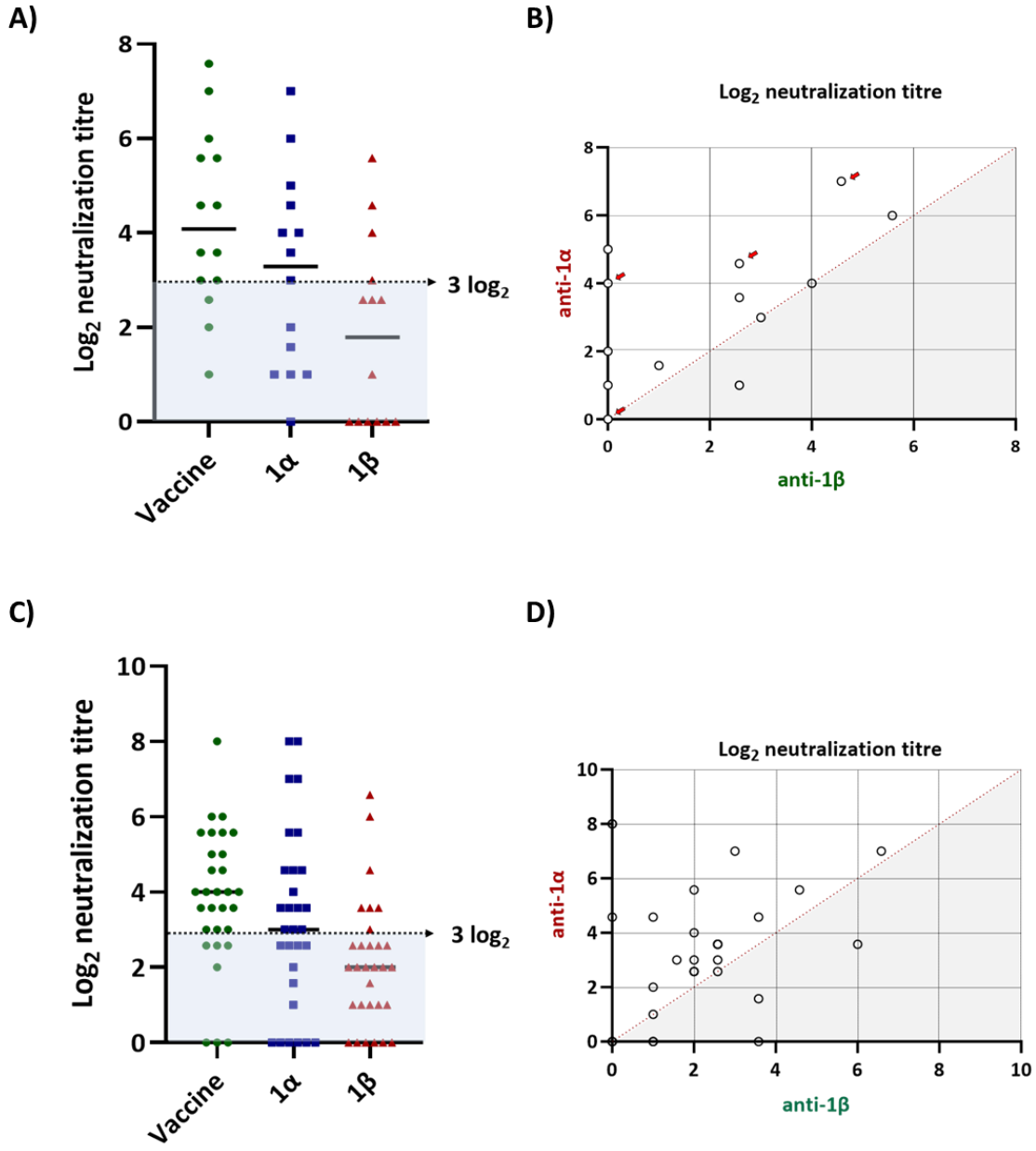

Figure 6. Neutralizing antibody titres in sows and piglets against the vaccine strain and the variants 1α and 1β. A) Distribution of neutralization titres of sows from Batch 1 against the vaccine virus, the variant 1α, and variant 1β. The shadowed area shows the individuals whose result was below 3 $\log_2$ for each tested viral isolate. B) Comparison of neutralization titres obtained for each sow from Batch 1 with variant 1α and variant 1β. The diagonal (dotted red line) represents the line of identity for both tests. Red arrows indicate sows that gave birth to viraemic animals. C) Distribution of neutralization titres of piglets at two weeks of age from Batch 1 against the vaccine virus, the variant 1α, and variant 1β. The shadowed area shows the individuals whose result was below 3 $\log_2$ for each tested viral isolate. D) Comparison of neutralization titres obtained for each piglet at two weeks of age from Batch 1 with variant 1α and variant 1β. The diagonal (dotted red line) represents the line of identity for both tests.

4.4.4. Replication kinetics of variants 1α and 1β in PAM.

The emergence of variant 1β corresponded with a decrease in the average C_t values of infected nursery pigs compared to C_t values when 1α was circulating, indicating an increase in the viral loads. To determine whether these differences were due to varying replication efficiencies, a replication kinetics experiment was performed. Figure 7 illustrates the replication kinetics of 1α and 1β in PAM over 72 hours. Notably, a significantly ($p<0.05$) lower C_t value (2.2 vs 2.9 $\log_{10}$) was observed at 12 hours post-infection in supernatants from 1β-inoculated cultures (Figure 7A). This was parallelled by a higher proportion of infected PAM at the same timepoint (Figure 7B). But the difference in C_t values and infection rates between the two variants did not persist at later time points (Figure 7A, C). Our results suggested that the superior replication capability of 1β contributed to the increased viral loads observed in nursery pigs.

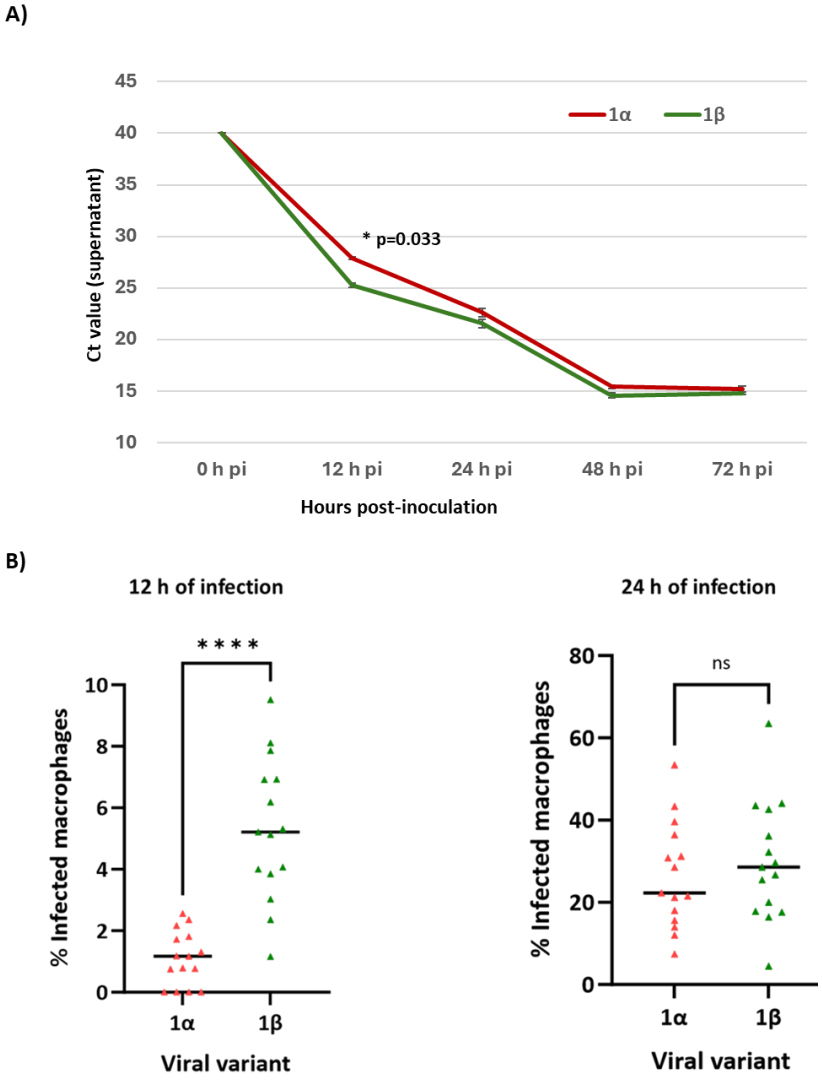

Figure 7. Results of the replication kinetics experiment for variants 1α and 1β. A) C_t values for the cell culture supernatants of PAM infected with variant 1α (red) and 1β (green) at MOI 0.1. B) Proportion of PAM labeling positive for PRRSV in the same cultures after 12 and 24 hours of incubation. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3.5. Attachment of variants 1α and 1β on PAM.

The findings of the replication kinetic experiments suggested that differences between variants 1α and 1β might stem from disparities in the initial stages of the viral replication cycle. For this purpose, an attachment experiment on PAM was performed. The results showed a greater number of 1β viral particles attaching on PAM compared to 1α (Figure 8), suggesting the enhanced attachment capability of 1β could be a contributing factor to its increased higher viral yields at early replication times.

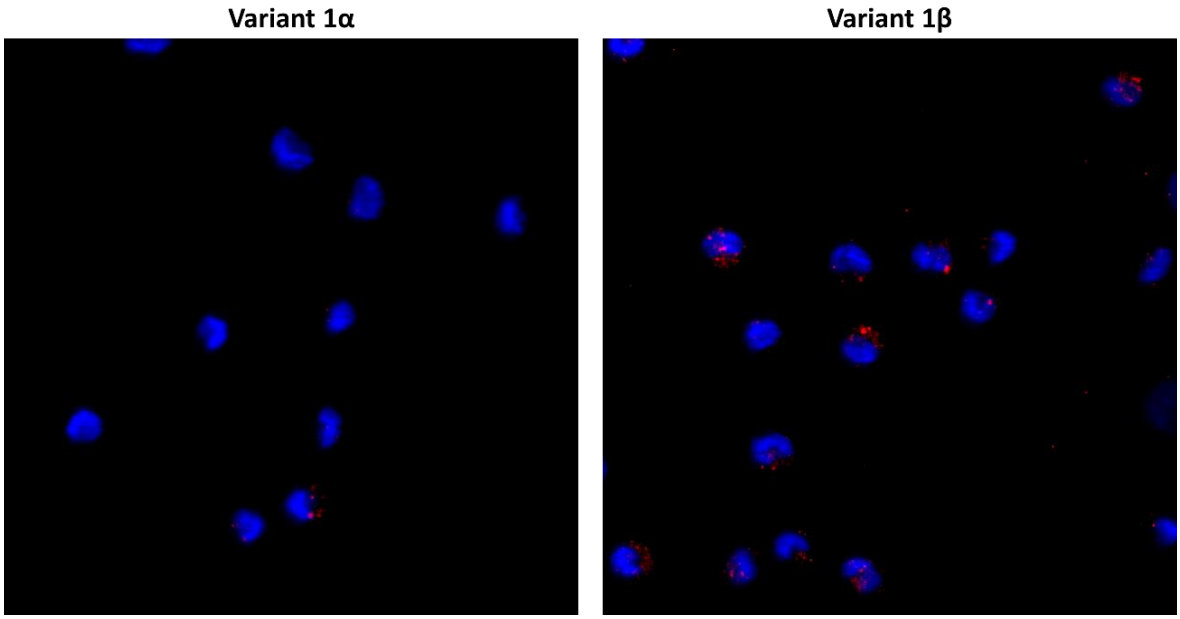

Figure 8. Attachment of variants 1α and 1β to PAM determined by confocal microscopy. The images show confocal microscopy z-projections of the attachment of variants 1α and 1β on PAM. PRRSV N protein was stained in red and PAM nuclei in blue.

4.4.6. Inhibition of poly I:C induced IFN-α and TNF-α.

Next, we explored the possibility that the emergence of variant 1β could have resulted from an enhanced capability to inhibit innate antiviral responses. For this purpose, the ability of both variants to inhibit IFN-α and TNF-α responses was examined using PAM cultures. When PAM were exposed to poly I:C either previously or simultaneously

with the virus, the release of IFN-α was reduced by more than 80% for both strains. Inoculating PAM with either the variant 1α or 1β prior to the addition of poly I:C led to a significant reduction of TNF-α levels, with no significant differences observed between the variants (Supplementary Figure 7).

4.5. DISCUSSION

Control and eradication of PRRSV stands as one of the utmost priorities for endemically infected farms. The strategies to achieve those goals are based on four pillars: monitoring of the infection, immunization of the herd, management of the pig flow, and biosecurity measures. However, despite the implementation of highly stringent protocols, certain farms are very difficult to stabilize, with infected animals being continuously found in the farrowing units or nurseries. For instance, Trevisan et al. (2019) reported that even following herd vaccination and interruption of replacement gilts entry, more than six months of closure were needed to confirm the complete cessation of viral circulation in 50% of the cases. Moreover, some farms required over a year of closure, while others consistently failed to achieve a negative status.

Certainly, there are several factors that contribute to the persistence of the virus within the herd which are well known and are shared by many communicable diseases. These include the introduction of replacement gilts without prior quarantine and vaccination, the mingling of susceptible and infected animals, and inadequate management practices that promote transmission through fomites, among others. Nonetheless, the role of the viral evolution in that context remains a scarcely explored area. The objective of the present work was to gain insight on how the virus evolves and persists within a farm under a strong pressure of vaccination.

The studied farm had been experiencing PRRSV circulation for years despite all efforts aimed at controlling the infection. At the commencement of the study, the farm was unstable, as evidenced by the detection of VTE occurring at birth. Somewhat surprisingly, the infection spread relatively slowly, with only 60% of the pigs being infected by the end of the nursery phase. Considering that maternally-derived antibodies usually wane around the fourth or fifth week of age (Andraud et al., 2018) and that piglets had not been vaccinated, a faster dissemination of the infection would have been anticipated.

Subsequently, in the next batch, this trend persisted, with only 36% of piglets infected in the nurseries. From then on, there were sharp increases in the incidence followed by a subsequent decline. The analysis of the obtained viral sequences shed light on the reasons behind this observed behaviour.

The phylogenetic analysis showed that during batches 0 to 5, a single viral strain, designated as strain 1, was circulating. Also, it was observed that during the sampling period of Batch 5, a distinct strain (nucleotide identity 83%), referred to as strain 2, was introduced into the farm from an external source. Upon closer examination of the results, it was also observed that strain 1 actually existed as two distinct variants, designated as 1α and 1β, which differed less than 0.5% of the nucleotide sequence. Notably, variant 1α was exclusively detected in batches 0 and 1 being subsequently replaced completely by variant 1β within a matter of a few weeks. This suggested that variant 1β had certain features that made it fitter for transmission within the context of a highly vaccinated farm.

The analysis of the C_t values of the animals infected in nurseries (lacking passive immunity) showed that the emergence of variant 1β correlated with a significant reduction in the average C_t values of infected nursery pigs. In other words, the viral loads of the grower pigs infected with 1β were higher compared to those infected with 1α in the previous batch, thereby suggesting again that variant 1β exhibited greater fitness than 1α within that particular context. Interestingly, in subsequent samplings, the average C_t values increased, indicating a decrease in viral loads. The introduction of strain 2 once again resulted in increased viral loads with the same pattern as above.

Moreover, there was a strong correlation between the average Ct of infected growers and the incidence in nurseries. Considering these findings collectively, it is plausible to hypothesize that variant 1β was fitter than 1α to replicate in pigs, consequently spreading faster in the nurseries. However, the subsequent decrease in the viral loads suggests that as the virus was transmitted to a larger number of animals over time, some form of selective constraint tended to limit viral replication within the studied context. The fact of observing this same behaviour with strain 2 indicates that this may be a generalized phenomenon in PRRSV-1. It is unlikely that this factor was immunity in the piglets since, in fact, most of them were infected when they had already lost maternally-derived antibodies.

One interesting finding in our study was that the emergence of variant 1β was not significantly related to an increase in the VTE or with the viral transmission in the farrowing units. The most reasonable explanation for this phenomenon is that most of the sows would have some level of immunity (either neutralizing antibodies or cell-mediated immunity) due to prior vaccinations or contact with strain 1α. Similarly, a high proportion of two-week-old piglets in batches 0 to 5 had biologically relevant titres of neutralizing antibodies which would certainly impede the transmission of the virus in the farrowing units. The introduction of strain 2 did result in an increase in VTE as expected, given its genetic distance to strain 1.

Once established that the emergence of a new variant, or the lateral introduction of a new strain, resulted in the rapid displacement of the former circulating variant, together with an increase in the incidence in the nurseries, the subsequent step entailed attempting to understand the mechanisms leading to this phenomenon. The comparison of the consensus sequences of 1α and 1β revealed that non-synonymous mutations were not randomly distributed, but rather accumulated in nsp1 (α and β), GP2, and GP3, and included one deletion in GP5, immediately following the known neutralization epitope in that protein. In arteriviruses, nsp1 has been reported to control the quantity of minus-strand templates for mRNA synthesis, thereby regulating replication (Nedialkova et al., 2010), but it also has an important role in inhibiting type I interferon responses in PRRSV-infected cells (Han and Yoo, 2014). GP2 and GP3, together with GP4, form a heterotrimer that interacts with CD163, the essential receptor for PRRSV (Calvert et al., 2007; Das et al., 2010; Van Gorp et al., 2010; Yu et al., 2020). Besides, GP5 interacts with porcine sialoadhesin, a co-receptor of PRRSV (Van Breedam et al., 2010), and it has been reported that GP5 may also interact with CD163 (Yu et al., 2020). Neutralizing antibodies against GP3, GP4, and GP5 have been documented in various studies (Vanhee et al., 2011), although contradictory reports also exist (Van Breedam et al., 2011; Li and Murtaugh, 2012). In addition, the neutralization epitope in GP4 is known to be very variable and may serve as a driving force in the selection of neutralization escape mutants (Costers et al., 2010b). Considering this background information and the epidemiological data, we hypothesized that 1β could potentially be an escape mutant of 1α with enhanced replication capability.

To evaluate that hypothesis, we assessed whether the sera of animals (sows and their piglets) that were present while 1α was circulating and before the emergence of 1β, had or not the same capability to neutralize both variants. The results indicated that, for most of the tested animals, neutralization titres were higher against 1α than against 1β. This supports the hypothesis that 1β was an escape mutant able to evade neutralization by the anti-1α antibodies. By employing a cut-off value of 3 $\log_2$ to consider the existence of homologous protection produced by neutralizing antibodies (Osorio et al., 2002; Lopez et al., 2007), it was observed that three out four sows, or a similar proportion of their offspring in the farrowing units, would not be protected against infection by 1β. Interestingly, this included some sows with titres as high as 5 $\log_2$ against 1α. The most likely explanation for these differences is that 1β was indeed a neutralization escape mutant, probably due to the mutations in the structural glycoproteins that are known to induce neutralizing antibodies. Moreover, these results also pointed specific amino acid positions in GP2, GP3, GP4, and GP5 that could be important targets for modifying the neutralizing characteristics of PRRSV-1 strains. Further laboratory investigations could delve into this topic. Besides, it is worth noting that both variants harboured double deletions in GP3 and GP4, with the latter encompassing the known neutralization epitope reported by Costers et al. (2010a). Additionally, 1β introduced a deletion in GP5. To our knowledge, this is the first report of a PRRSV-1 isolate presenting simultaneously multiple deletions in all those structural glycoproteins.

Regarding strain 2, the dissemination within the farm can be explained simply by the genetic distance and the lack of specific neutralizing antibodies in the sows. It is well known that, in most cases, the neutralizing antibodies induced by one PRRSV-1 strain are little reactive against other genetically distant counterparts (Martínez-Lobo et al., 2011).

As previously mentioned, the rapid spread of variant 1β and the increased viral loads suggested that this variant had a better replication fitness in comparison to variant 1α. In the replication kinetic experiments, 1β produced a higher yield of virus and a higher proportion of infected cells within the initial twelve-hour period of incubation, but not in later time intervals. This fact indicates that the observed differences could possibly be attributed to better attachment or internalization capabilities. Accordingly, an experiment was set up to test this phenomenon. The results showed that 1β exhibited a higher degree of attachment on PAM compared to 1α, resulting in increased replication in the cells, most

likely due to the internalization of a larger number of viral particles. Obviously, this would confer an advantageous feature to 1β. It is tempting to postulate that this increased ability to infect PAM and the resulting increased viral loads would culminate in an enhanced viral shedding, thus accelerating the transmission rate of 1β. The underlying mechanism behind this would point again to GP5, as it interacts with porcine sialoadhesin on the surface of PAM (Van Breedam et al., 2010) and possibly with CD163, and GP2 and GP3, due to their interaction with CD163 (Yu et al., 2020). Once again, the observed mutations and deletions may indicate the presence of critical residues within those proteins.

We finally examined whether 1β had a higher capability to regulate the production of innate antiviral cytokines (IFN-α and TNF-α) in inoculated PAM. The results indicated that although both strains strongly downregulated the IFN-α and TNF-α responses following TLR-3 stimulation, no statistically significant differences were observed. This suggests that this mechanism was not implicated in the emergence of variant 1β.

An intriguing question is how the variant 1β was originated. Several facts must be considered: first, the mutations fixed in the 1β variant were already present as minor variants within the 1α quasispecies of some individuals; second, within an individual, the increase in one mutation was not related to the increase in the frequency of another mutation, except within nsp1α; third, there is evidence of recombination between 1α and 1β; and fourth, the replacement of 1α was extremely fast and it disappeared from the population in just few weeks. Taken together, it seems plausible that at one point several 1α subvariants, each one harbouring one or more advantageous mutations, underwent recombination to produce the founder 1β variant. The alternative notion of a gradual selection of up to twenty-five mutations to produce 1β is difficult to reconcile with these facts.

At this point, it is possible to draw a picture of how farms may persist endemically infected over prolonged periods of time. Our study showed that, besides the occurrence of lateral introductions of the virus, a combination of evolutionary events may result in the emergence of variants that accumulate several mechanisms to gain fitness in an immune, or partially immune, population. These mechanisms include the evasion of neutralizing antibodies and enhanced capability to infect PAM. Whether these two mechanisms are related to the same mutations or not cannot be definitively inferred from the present study. However, this can be experimentally evaluated in further studies.

Nevertheless, several unresolved inquires remain. The first one pertains to the origin of variant 1β, whether it emerged in sows or piglets. Another question that deserves further investigation is why after the emergence of a variant (or the introduction of a new strain) and the initial increase in the incidence, there was a subsequent decrease. Unfortunately, we are unable to postulate an explanation based on evidence for these facts.

The current study highlights the plasticity of PRRSV and the several mechanisms that the virus can use to persist in the population despite intensive vaccination protocols. Moreover, this also reveals that as long as the virus continues to circulate in the population, it will be very difficult to avoid the emergence of novel variants with enhanced capability for infecting pigs. Consequently, this points towards the need for developing newer and more efficacious vaccines, as well as to implement eradication programs with all available resources.

Data availability

The datasets are available GenBank and accession numbers can be found in the main text or the supplementary materials.

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Conflict of interest

The authors declare that they have no competing interests.

4.6. SUPPLEMENTARY MATERIAL

Supplementary Table 1. List of oligonucleotide primers (F: forward primer, R: reverse primer) used for amplifying PRRSV-1 ORF5 and the size of the amplicon.

Gene	Sequence
ORF5	
Forward L1F	5′- TGAGGTGGGCTACAACCATT -3′
Reverse C1	5′- GCGTGACACCTTAAGGGC -3′
Reverse C6	5′- GCGACACCTTAAGGGCATATATCA -3′

Supplementary Table 2. PRRSV-1 complete genome sequences retrieved from GenBank for phylogenetic analysis. Lelystad virus is the prototype for PRRSV-1. Contemporary strains in the farm's geographical zone (underlined) and the five licensed vaccines in Spain (in red) are included. These PRRSV-1 strains were employed for comparative analyses in Supplementary Table 5, Figures 2, and 3. Adapted from Clilverd et al., (2023).

Accession Number	Strain ID	Country	Year
NC043487	***Lelystad virus***	Netherlands	1993
KF203132	*Olot/91*	Spain	1991
JF276431	*CReSA3262*	Spain	1992
KX249748	*CReSA3*	Spain	2013
KX249755	*CReSA228*	Spain	2013
KX249756	*CReSA261*	Spain	2013
KX249753	*CReSA100*	Spain	2014
OP688199	*B1-522-4w*	Spain	2017
OP688221	*B3-785-9w*	Spain	2018
OP822977	*Nu4a*	Spain	2021
OP822964	*M3*	Spain	2021
OP822963	*N5*	Spain	2021
OP822973	*Nu1*	Spain	2021
OM893828	*R1*	Spain	2021
OM893829	*R2*	Spain	2021
JF276435	*CReSA3267*	Portugal	2006
KT326148	*AUT13-883*	Austria	2013
JF802085	*Lena*	Belarus	2007
KP889243	*SU1-Bel*	Belarus	2010
GU737264	*07V063*	Belgium	2007
KT159248	*13V091*	Belgium	2013
KT159249	*13V117*	Belgium	2013
GU047344	*BJEU06-1*	China	2006
MN927227	*HeB3*	China	2018
KC862567	*DK-2011-0511-14*	Denmark	2011
KY767026	*FR-2014-56-11-1*	France	2014
MH018883	*FR-2016-56-11-1*	France	2016
MH463457	*HU19401*	Hungary	2016
MF346695	*PR40-2014*	Italy	2014
KX668221	*WestSib13*	Russia	2013
KY434183	*CBNU0495*	South Korea	2016
GU067771	***Unistrain®PRRS***	-	-
KJ127878	***Porcilis® PRRS***	-	-
LQ787782	***Suvaxyn® PRRS***	-	-
GQ461593	***PYRSVAC-183®***	-	-
KT988004	***PRRSFlex®EU***	-	-

Supplementary Table 3. Amino acid mutations resulting from the adaptation of PRRSV-1 isolates to MARC145-cells. The table displays the accumulated non-synonymous amino acid mutations across the genome resulting from the adaptation after four cellular passages of an isolate of variants 1α and 1β and strain 2 to MARC145-cells. Nucleotide position correspond to the alignment with the prototype PRRSV-1 strain Lelystad (LV; NC_043487). Amino acids are represented with a one-letter code. The first letter indicates the amino acid in the isolate, the number the position in the protein, and the second letter the amino acid in isolate adapted to MARC-145 cells.

Strain/Virus	Protein	Amino acid mutation
Variant 1α	**nsp7b**	S51P
	nsp9	F533L
	nsp10	R429G
	GP2	V170A
	GP4	I67Del
Variant 1β	**nsp2**	H499Q
		S506R
		I509L
		P518Q
Strain 2	**nsp2**	Y226C
	nsp10	P281L

Supplementary Table 4. Similarities (p-distance ± standard error) within and between variant 1α, 1β, and strain 2. The table presents the average nucleotide differences within and between the different variants of PRRSV-1 whole genome consensus sequences and individual segments of the viral genome. nsp = non-structural protein.

Segment	Variant 1α	Variant 1β	Strain 2	1α vs. 1β	1α vs. 2	1β vs. 2
Genome	0.0034±0.0003	0.0029±0.0002	0.0092±0.0005	0.0046±0.0053	0.1676±0.0031	0.1687±0.0031
ORF1a						
nsp1α	0.0034±0.0013	0.0034±0.0012	0.0134±0.0028	0.0014±0.0047	0.1355±0.0145	0.1276±0.0140
nsp1β	0.0051±0.0012	0.0020±0.0005	0.0080±0.0019	0.0066±0.0031	0.2133±0.0161	0.2215±0.0164
nsp2	0.0046±0.0007	0.0048±0.0008	0.0129±0.0012	0.0035±0.0010	0.2023±0.0077	0.2021±0.0077
nsp3	0.0026±0.0008	0.0025±0.0008	0.0110±0.0020	0.0062±0.0026	0.1812±0.0127	0.1860±0.0129
nsp4	0.0027±0.0008	0.0025±0.0010	0.0083±0.0022	0.0035±0.0022	0.1792±0.0156	0.1783±0.0157
nsp5	0.0043±0.0016	0.0025±0.0010	0.0050±0.0017	0.0020±0.0013	0.1751±0.0170	0.1765±0.0170
nsp6	0.0021±0.0020	0.0000±0.0000	0.0096±0.0093	0.0000±0.0000	0.1688±0.0547	0.1688±0.0547
nsp7a	0.0020±0.0008	0.0038±0.0014	0.0076±0.0023	0.0053±0.0033	0.1543±0.0173	0.1553±0.0171
nsp7b	0.0063±0.0024	0.0035±0.0015	0.0112±0.0030	0.0048±0.0029	0.1748±0.0199	0.1713±0.0200
nsp8	0.0029±0.0028	0.0004±0.0004	0.0152±0.0054	0.0038±0.0039	0.1541±0.0303	0.1537±0.0303
ORF1b						
nsp9	0.0022±0.0006	0.0019±0.0005	0.0052±0.0009	0.0027±0.0011	0.1451±0.0079	0.1457±0.0079
nsp10	0.0036±0.0009	0.0027±0.0007	0.0074±0.0014	0.0047±0.0018	0.1641±0.0099	0.1662±0.0100
nsp11	0.0016±0.0006	0.0022±0.0009	0.0078±0.0019	0.0005±0.0005	0.1562±0.0139	0.1564±0.0138
nsp12	0.0032±0.0015	0.0032±0.0012	0.0094±0.0027	0.0068±0.0035	0.1727±0.0168	0.1762±0.0169
ORF2a	0.0024±0.0009	0.0009±0.0003	0.0110±0.0023	0.0085±0.0032	0.1456±0.0128	0.1514±0.0131
ORF2b	0.0005±0.0005	0.0017±0.0010	0.0094±0.0036	0.0047±0.0047	0.0799±0.0187	0.0838±0.0192
ORF3	0.0051±0.0012	0.0033±0.0009	0.0009±0.0019	0.0059±0.0024	0.1611±0.0128	0.1602±0.0128
ORF4	0.0014±0.0007	0.0029±0.0010	0.0090±0.0023	0.0053±0.0030	0.1377±0.0151	0.1406±0.0151
ORF5a	0.0060±0.0044	0.0034±0.0020	0.0087±0.0042	0.0028±0.0026	0.1382±0.0285	0.1372±0.0288
ORF5	0.0039±0.0010	0.0032±0.0009	0.0114±0.0023	0.0043±0.0024	0.1760±0.0152	0.1780±0.0154
ORF6	0.0010±0.0004	0.0021±0.0011	0.0145±0.0023	0.0045±0.0027	0.1157±0.0139	0.1180±0.0139
ORF7	0.0028±0.0015	0.0007±0.0004	0.0052±0.0021	0.0061±0.0038	0.1070±0.0149	0.1065±0.0150

Supplementary Table 5. Similarities (p-distance) between the circulating viruses in this study and the reference PRRSV-1 strains. The table depicts the nucleotide mean differences among the examined variants of PRRSV-1 whole genome consensus and ORF5 sequences and the sequences obtained from GenBank.

	Genome			ORF5		
PRRSV-1 strain	1α	1β	2	1α	1β	2
***Lelystad virus*-NC043487**	0,1314	0,1361	0,1278	0,1486	0,1488	0,1151
Olot/91-KF203132	0,1285	0,1336	0,1376	0,1386	0,1388	0,1220
CReSA3-KX249748	0,1605	0,1632	0,1443	0,1572	0,1575	0,1394
CReSA228-KX249755	0,1317	0,1369	0,1398	0,1405	0,1407	0,1222
CReSA261-KX249756	0,1090	0,1202	0,1705	0,1386	0,1371	0,1598
CReSA100-KX249753	0,1067	0,1133	0,1579	0,1586	0,1605	0,1615
B1-522-4w-OP688199	0,1377	0,1474	0,1669	0,1636	0,1672	0,1649
B3-785-9w-OP688221	0,1387	0,1483	0,1680	0,1619	0,1639	0,1615
Nu4a-OP822977	0,1034	0,1126	0,1714	0,1820	0,1839	0,1529
M3-OP822964	0,1739	0,1800	0,1780	0,1636	0,1622	0,1564
N5-OP822963	0,1445	0,1519	0,1530	0,1586	0,1589	0,1409
Nu1-OP822973	0,1711	0,1768	0,1729	0,1736	0,1756	0,1735
R1-OM893828	0,1896	0,1957	0,1860	0,1803	0,1856	0,1701
R2-OM893829	0,1897	0,1957	0,1859	0,1803	0,1856	0,1701
CReSA3267-JF276435	0,1313	0,1363	0,1383	0,1419	0,1421	0,1203
AUT13883-KT326148	0,1677	0,1730	0,1682	0,1553	0,1555	0,1478
Lena-JF802085	0,2047	0,2148	0,2097	0,2020	0,2007	0,1615
SU1Bel-KP889243	0,2089	0,2162	0,2092	0,1870	0,1940	0,1804
07V063-GU737264	0,1593	0,1673	0,1591	0,1603	0,1622	0,1357
13V091-KT159248	0,1704	0,1752	0,1686	0,1987	0,1973	0,1735
13V117-KT159249.1	0,1597	0,1678	0,1592	0,1603	0,1622	0,1357
BJEU061-GU047344.1	0,1556	0,1616	0,1535	0,1653	0,1672	0,1375
HeB3-MN927227	0,1567	0,1621	0,1531	0,1703	0,1689	0,1392
DK2011051114-KC862567	0,1389	0,1438	0,1353	0,1572	0,1608	0,1291
FR201456111-KY767026	0,1333	0,1393	0,1386	0,1452	0,1455	0,1289
FR201656111-MH018883	0,1367	0,1433	0,1423	0,1436	0,1455	0,1340
HU19401-MH463457	0,1648	0,1720	0,1678	0,1703	0,1689	0,1615
PR40l2014-MF346695	0,1813	0,1842	0,1778	0,1820	0,1823	0,1340
WestSib13-KX668221	0,2111	0,2204	0,2122	0,2154	0,2174	0,2285
CBNU0495-KY434183	0,1681	0,1728	0,1723	0,1753	0,1789	0,1564
***Porcilis PRRS*-KJ127878**	0,1326	0,1367	0,1281	0,1519	0,1522	0,1237
***PRRSFlex EU*-KT988004**	0,1572	0,1649	0,1548	0,1469	0,1472	0,1340
***Unistrain*-GU067771**	0,1315	0,1367	0,1391	0,1452	0,1455	0,1237
***Suvaxyn PRRS*-LQ787782**	0,1518	0,1560	0,1503	0,1753	0,1756	0,1237
***PYRSVAC183*-GQ461593**	0,1315	0,1369	0,1391	0,1469	0,1472	0,1271

Supplementary Table 6. Amino acid comparison of PRRSV-1 sequences variants 1α, 1β, and strain 2. The table shows the differences between the predicted amino acid composition of the different viral proteins inferred from the consensus nucleotide sequences of the variants 1α, 1β, and strain 2 (in rows). Columns indicate positions where amino acid differences were found in each examined protein. The amino acid position corresponds to the alignment with the prototype PRRSV-1 strain Lelystad (LV; NC_043487). Grey shaded cells show the position where a different amino acid was found between variants 1α and 1β. Yellow shaded cells indicate insertions or deletions.

nsp1α (n=18)

Amino acid position	24	36	42	55	58	71	79	84	95	112	113	114	127	138	140	142	162	169
Strain 1α	F	S	L	N	H	T	S	I	T	S	R	Q	D/N	V	V	S	L	C
Strain 1β	Y	·	P	·	Q	·	A	·	·	·	·	·	D	·	·	A	·	·
Strain 2	Y	P	T	D	H/R	S	·	L	L	A	S	R/K	D	M	L	A	S	R/C

nsp1β (n=40)

Amino acid position	8	9	13	16	17	18	21	26	28	30	33	34	37	43	46	50	60	62	67	69	72	77	78
Strain 1α	G	L	V	T	L	D	S	T	G	S	A	S	V	G	A	T	N	A	T	T	S	S	M
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	N	S	M	L	R	N	T	A	E	N	V	N	A	I	V	I	D/N	S	V	S	H	G	T

Amino acid position	80	82	85	87	102	105	106	107	109	114	117	159	170	180	186	191	195
Strain 1α	Q	C	L	Q	F	P	A	E	W	H	G	P	E	M	S	V	V
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	F
Strain 2	H	S	I/V	T	L	S	P	G	A	Y	N	S	G	M	P	T	V

nsp2 (n=209)

Amino acid position	8	9	12	14	15	16	18	19	20	21	25	26	28	31	33	34	40	48	53	58	66	69
Strain 1α	A	R	V	N	D	K	S	V	T	A	A	K	I	C	A	T	T	V	V	H	M	D
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	N
Strain 2	T	K	A	S	K	G	S/L	T	A	S	V	P	I/T	Y	T	V	A	V/A	M	N	A	N

Amino acid position	72	78	82	83	88	98	107	108	122	132	133	143	147	148	149	151	152	160	170	174
Strain 1α	P	D	A	E	M	R	I	K	V	I	V	P	E	G	E	S	E	A	V	T
Strain 1β	·	·	·	·	·	·	·	R	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	E	D/C	T	Q	L	H	I/M	K	M	V	I	S	D	D	G	P	N	S	S	S

Amino acid position	178	182	184	187	200	203	230	231	239	248	249	262	268	270	272	274	275	276	278
Strain 1α	A	P	G	S	S	T	R	M	K	V	R	E	V	A	L	P	T	S	D
Strain 1β	·	·	·	·	S/P	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	N	Del	E	T	S	S	K	L	R/K/G	I	K	D	I/T	S	S	L	A	P	E

Amino acid position	280	282	284	285	289	288-298	300	301	302	303	305	306	308	309	311	313	315
Strain 1α	S	S	D	T	L		V	A	S	Q	A	Q	S	S	E	A	S
Strain 1β	·	·	·	·	L/S		·	·	·	·	·	·	·	·	·	·	·
Strain 2	P	N	G	A	A	Del	A	T	P	E	V	P	G	G	K	V	L

Amino acid position	317	319	320	321-358	362	363	364-490	406	420	436	437	491	492	497	501	504	509
Strain 1α	V	V	A	Del	G	G	Del	-	-	-	-	G	H	R/L	G	G/E	I
Strain 1β	·	·	·	·	·	·	·	-	-	-	-	·	·	·	·	·	·
Strain 2	P	T	K	-	S	E/G	-	G/S	P/S	G/E	S/L	S	D	L	E	E	T

Amino acid position	511	514	516	517	519	520	523	525	527	528	529	532	533	534	535	536	539
Strain 1α	L	M	D	A	V	A	S	G	T	T	P	V	S	S	G	A	L
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	F	A	G	V	I	V	P	D	T/A	I	S	A	L	Q	E	V	F

Amino acid position	544	545	549	563	567	570	573	592	595	599	622	637	638	642	643	644	646
Strain 1α	L	K	Y	D	R	N	Y	D	N	E	K	R	K	N	D	C/Y	D
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	P	N	F	G	K	S	F	A	D	N	D	K	K/R	S	S	N	V/I

Amino acid position	649	657	659	(660)	660	661	662	664	666	667	668	670	671	672	673	674	681
Strain 1α	Y	K	S		V	V	S	P	P	V	E	A	P	G	R	A	I
Strain 1β	·	·	·		·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	Q	K/R	G	Ins G	P	L/P	P	Q	S	T	G	V	F	N	Q	T	T

Amino acid position	682	683	684	685	686	687	688	689	691	694	695	697	698	699	702	703	704
Strain 1α	P	L	E	G	V	T	T	P	G	R	A	G	L	P	V	D	T
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	R	Q	G	N	T	A	P	L	M	H	V	D	P	G	A	G	E

Amino acid position	705	706	710	711	712	713	716	720	721	722	723	726	727	729	734	741	750
Strain 1α	G	G	D	R	M	L	A	E	L	V	G	L	T	R	F	M	V
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A
Strain 2	G/E	G/R	N	L	V	I	T	G	S	I	S	S	M	W	V	A	S

Amino acid position	753	761	765	768	770	771	774	788	812	824	836	843	852	856	858	865	869
Strain 1α	S	V	V	F	R	L	A	V	D	K	Q	G	S	F	R	H	I
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	P	I	A	L	Y/H	S	I	L	N	E	E	T	L	L	K	Y	V

Amino acid position	871	881	883	884	920	931	945	949	957	988	1003	1005
Strain 1α	L	L	I	I	A	S	H	C	S	I	L	T
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	F	F/L	L	V	T	A	R	R	P	V	Q	V

nsp3 (n=34)

Amino acid position	12	18	21	39	54	58	59	60	63	64	65	75	76	91	102	107	117	123	127	137	139
Strain 1α	N	N	V	T	I	S	S	T	R	A	T	A	Q	F	P	S	P	P	A	Y	A
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	K	E	I	A	T	N	P	A	K	T	V	V	H	L	A	A/T	P/S	V	F	T	I

Amino acid position	152	156	159	160	172	179	180	185	215	223	234	235	292
Strain 1α	V	I	T	A	F	G	G	M	V	V	V	T	I
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	I	V	V	T	L	C	A	A/V	M	I	A	V	V

nsp4 (n=29)

Amino acid position	6	10	31	33	34	38	45	52	55	58	60	69	70	72	74	76	79	81	82	83	85	105
Strain 1α	R	P	K	I	V	T	A	S	R	T	R	D	A	N	Q	V	A	K	V	V	G	E
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	N/D	·	·	·	·	·	·	·	D
Strain 2	H	L	R	T	I	A	T	E	C	T/V	K	H	V	N/D	P	I	V	N	I	A	R	N

Amino acid position	113	129	147	178	180	188	201
Strain 1α	S	I	D	G	T	I	V
Strain 1β	·	·	·	·	·	·	·
Strain 2	N	V	E	S	A	V	T/I

nsp5 (n=14)

Amino acid position	26	37	57	86	89	98	102	105	120	122	148	151	152	158
Strain 1α	V	I	L	L	I	T	R	D	I	M	H	A	M	A
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	I	V	V	F	V	V	K	E	L	V	C	D	T	H

nsp6 (n=1)

Amino acid position	5
Strain 1α	R
Strain 1β	·
Strain 2	K

nsp7a (n=10)

Amino acid position	20	51	65	67	79	106	116	134	138	139
Strain 1α	S	R	V	S	S	R	N	N	D	V
Strain 1β	·	·	·	·	P	·	·	·	·	·
Strain 2	N	Q	I	A	S	K	S	D	G	I

nsp7b (n=17)

Amino acid position	7	26	28	37	48	53	57	64	65	69	76	77	78	88	90	94	110
Strain 1α	C	F/L	S	V	T	Y	Y	A/V	N	R	V	T	S	T	A	I	I
Strain 1β	·	F	N	·	·	·	·	A	·	·	·	·	·	·	·	·	·
Strain 2	S	F	N	I	N	Y/F	H	A	D	K	I	D	H	I	V	V	V

nsp8 (n=3)

Amino acid position	3	32	39
Strain 1α	R	D	T
Strain 1β	·	·	·
Strain 2	K	N/D	T/N

nsp9 (n=31)

Amino acid position	1	2	29	49	51	55	66	106	109	119	148	157	158	162	169	178	188	192	221	232
Strain 1α	T	S	V	V	V	T	S	D	I	V	Y	H	A	T	L	G	H	A	M	P
Strain 1β	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·
Strain 2	A	G	I	A	I	I	C	D/N	V	A/V	H	Y	Q	I	I	R/K	Q	V/A	V	F/L

Amino acid position	234	241	270	311	339	413	465	487	543	557	584
Strain 1α	F	R	V	I	L	A	I	G	T	R	A
Strain 1β	·	K/R	·	·	·	·	·	G/S	·	·	·
Strain 2	C	K	I	V	P	V	V	G	K	K	T

nsp10 (n=32)

Amino acid position	2	9	16	40	47	51	59	61	64	66	84	89	116	180	182	216	217	261	266	325	326
Strain 1α	R	V	H	N	A	S	A	K	I	N	C	V	P	V/I	S	Y	T	Y	D	I	G
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	V	·	·	·	·	·	·	·
Strain 2	K	I	Y	S	S	P	T	R/K	L	A	S	A	A	I	C	H	V	C	N	T	G/D

Amino acid position	341	354	375	378	382	387	404	406	420	428	429
Strain 1α	I	K	N	Q	D	R	N	D	S	L	R
Strain 1β	·	·	·	K	·	·	·	·	·	P	·
Strain 2	V	R	D	Q	N	H	D	D/N	P	P	K

nsp11 (n=10)

Amino acid position	23	40	59	71	76	92	98	119	120	155
Strain 1α	A	H	G	V	T	I	A	T	A	F
Strain 1β	·	·	·	·	·	·	·	·	·	·
Strain 2	V	S	A	A	I	V/I	G	A	T	T

nsp12 (n=20)

Amino acid position	2	42	57	59	81	83	84	88	97	100	106	115	118	138	145	146	147	148	149	151
Strain 1α	L	D	Q	V	R	S	L	S	R	N	I	N	F	Q	Q	L	P	P/S	E	E
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	R	·	·	·	·	·	·
Strain 2	F	N	K	I	K/R	T	F	N	C	D	T	E	C	Q	R	S	L	L	G	A

GP2 (n=35)

Amino acid position	8	11	13	16	22	24	25	26	27	29	30	32	36	41	42	67	80	83	88	98	102	106
Strain 1α	V	A	C	M	S	A	W	L	T	S	F	L	L	P	D	S	R	A	F	E	H	E
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	K	·	·	·	R	D
Strain 2	A	P	Y	T	L	V	W/L	S	I	L	F/S	W	L	Q	V	N	R	V	I	M	H	E

Amino acid position	135	138	141	170	177	181	187	192	194	232	235	244	248
Strain 1α	K	R	I	V	L	T	L	S	S	A	Y	V	S
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	R	N	V	A	Q	S	I/L	P	T	V	C	A	L

E (n=3)

Amino acid position	9	61	69
Strain 1α	S	V	I
Strain 1β	·	·	·
Strain 2	T	I	V

GP3 (n=58)

Amino acid position	3	4	7	10	11	12	14	15	18	23	30	46	48	60	64	76	80	81	91	92	93	100
Strain 1α	Y	Q	R	F	L	L	S	F	Y	A	T	Q	A	R	M	R	D	H	L	M	P	D
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	N
Strain 2	H	K	C	F/L	F	F	G	L	H	T	F/S/T	E	T	S	Y/H	K	T	T	S	L	S	N

Amino acid position	102	127	139	140	142	146	154	156	157	158	166	172	204	207	211	215	216	217	218
Strain 1α	R	G	G	R	A	E	V	A	N	H	V	I	A	A	I	F	K	P	I
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	K	G/R	R	H	F	V	I	T	T	Y	A	V	V	V	T	L	R	L	T

Amino acid position	224	232	234	235	236	237	239	244	245	247	248	250	252	261	262	263	265
Strain 1α	V	S	S	D	I	Del	S	Del	K	L	L	N	H	L	N	T	R
Strain 1β	·	·	P	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	G	I	S	N	H	T	P	G	A	P	Del	Y	P	P	S	I	Q

GP4 (n=28)

Amino acid position	8	11	12	14	29	35	50	51	53	54	55	57	58	63	64	65	66	67	68	69	71	128
Strain 1α	F	S	T	Y	P	Q	I	E	L	R	Del	W	V	E	G	Del	A	I	R	K	S	T
Strain 1β	·	·	·	·	·	·	·	·	P	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	L	G	A	H	T	K	L	D	·	Q	P/L	R	A	R	S	S	P	Del	G	V	A	V

Amino acid position	143	149	150	154	162	179
Strain 1α	I	Y	H	D	L	I
Strain 1β	·	·	·	·	·	·
Strain 2	T	H	Y	N	M	V/I

GP5 (n=40)

Amino acid position	2	4	6	7	9	11	13	14	17	18	20	22	25	31	33	35	46	56	58	59	60	79	93
Strain 1α	R	L	K	L	L	S	L	H	F	W	F	F	I	S	D	S	N/D	N	L	F	G	I	L
Strain 1β	·	·	·	·	·	·	·	·	P	·	·	·	·	·	·	·	N	·	Del	·	·	·	·
Strain 2	K	S	R	S	H	L	P	Y	C	C	L	L	T	F	V	N	N	A	L	R/H	K	L	F

Amino acid position	96	97	101	104	105	106	115	122	123	125	130	162	166	173	174	182	193
Strain 1α	G	A	I	Y	D/G	G	C	L	T	L	V	V	S	S	N	V	R
Strain 1β	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	S	V	A	H	D	G/R	G	F	V	F	A	I	L	G	D	I	K

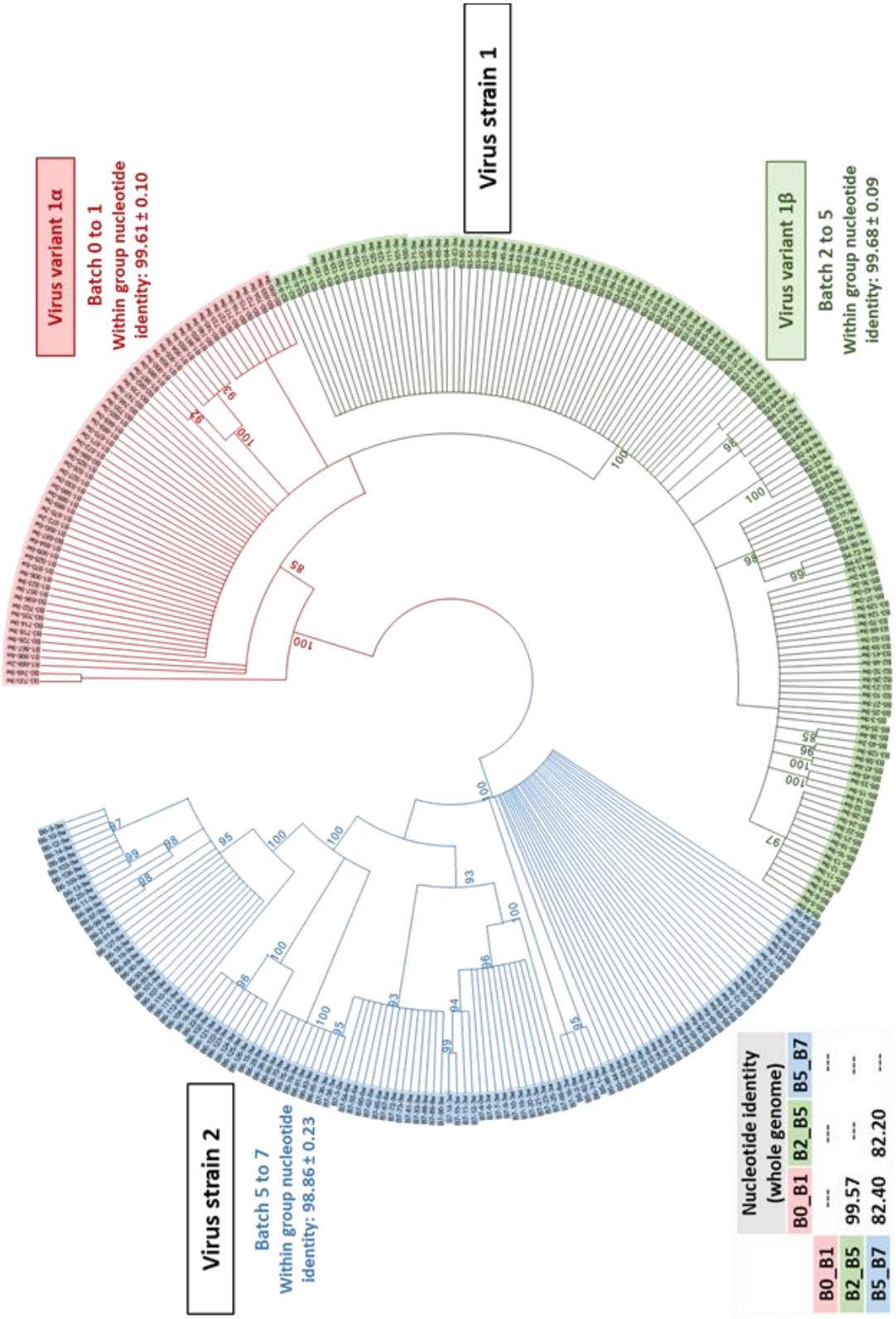
M (n=20)

Amino acid position	3	6	9	11	14	18	28	62	65	74	80	84	87	94	100	123	130	152	161	165
Strain 1α	S	N	G	P	A	V	I	A	E	L	F	V	F	V	M	P	R	S	V	L
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	G	N/D	Y	S	V	A	V	E	Q	M	L	I	L	I	L	S	Q	G	V/I	V

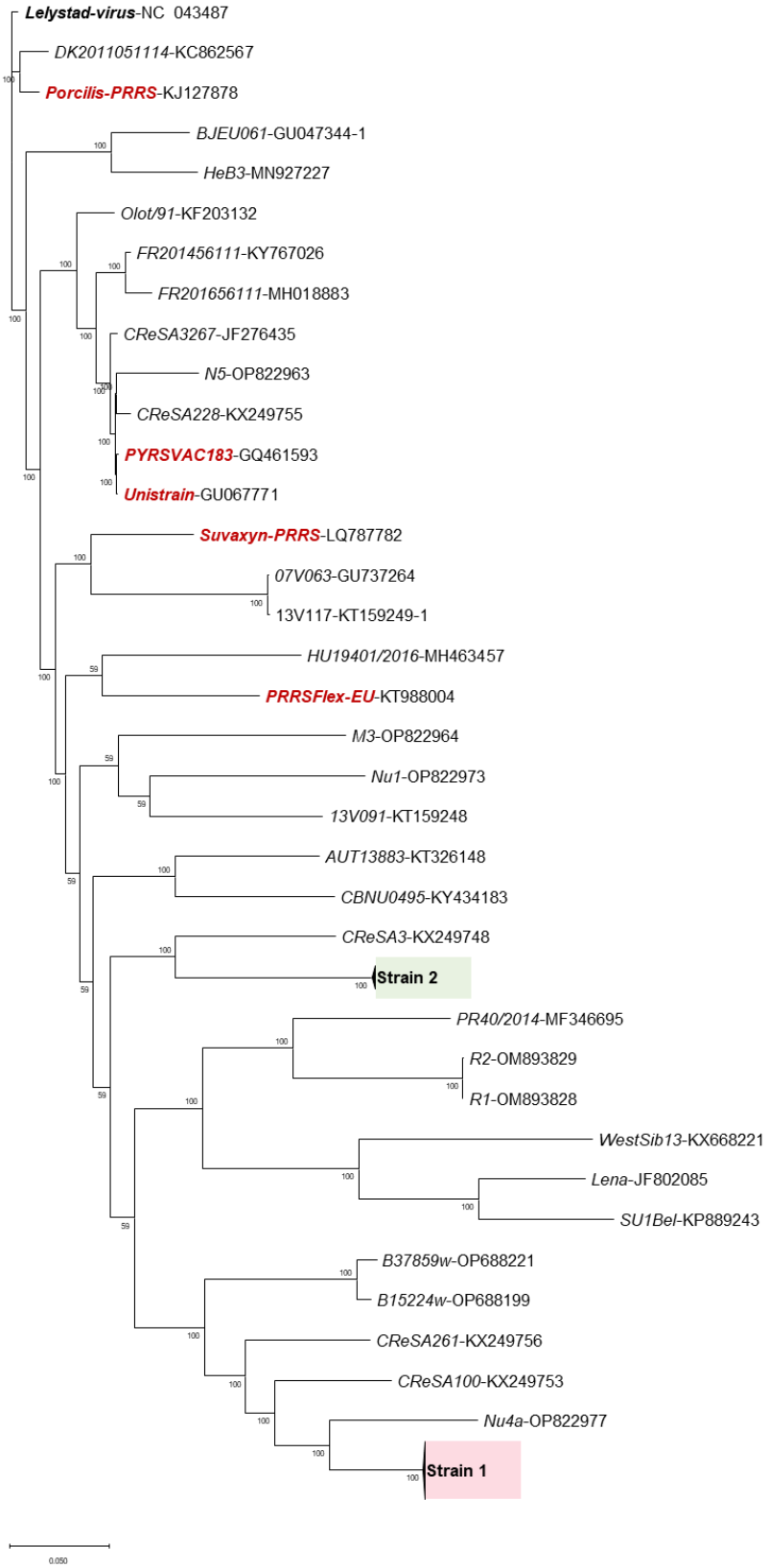
N (n=17)

Amino acid position	7	10	12	20	23	33	34	35	40	41	70	90	100	122	126	127	128
Strain 1α	G	N	Q	S	I	V	M	R	R	P	L	A	S	S	N	V	D
Strain 1β	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Strain 2	S	K	K	G	V	M	I	K	Q	S	Q	V	G	S/P	G	A	N

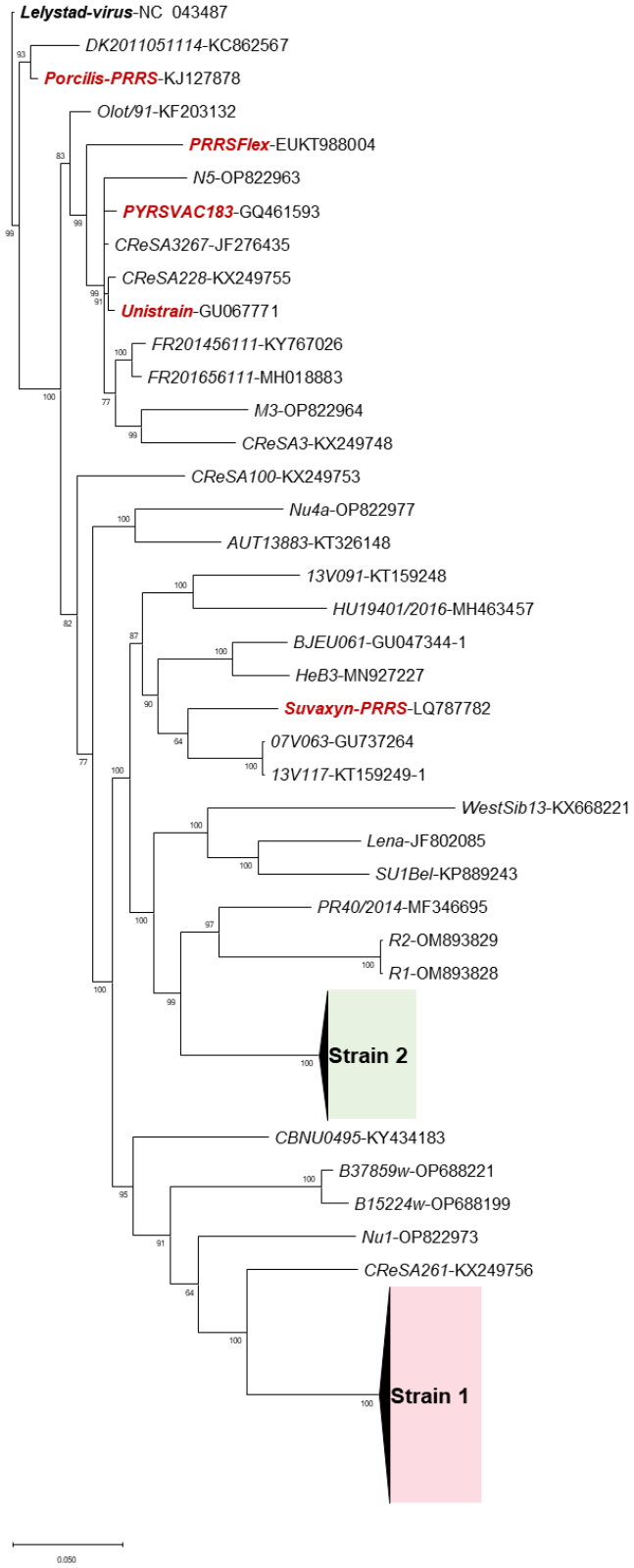
Supplementary Figure 1. Bayesian tree showing the phylogenetic grouping of the ORF5 sequences obtained in this study. Posterior probabilities higher than 70% are shown. All isolates from batches 0 and 1 belonged to the 1α cluster (red). All isolates of batches 2 to 4 and most of the Batch 5 sequences belonged to variant 1β (green). In Batch 5, a new strain appeared in the nurseries and subsequently became the only detectable PRRSV strain in batches 6 and 7 (strain 2, in blue).



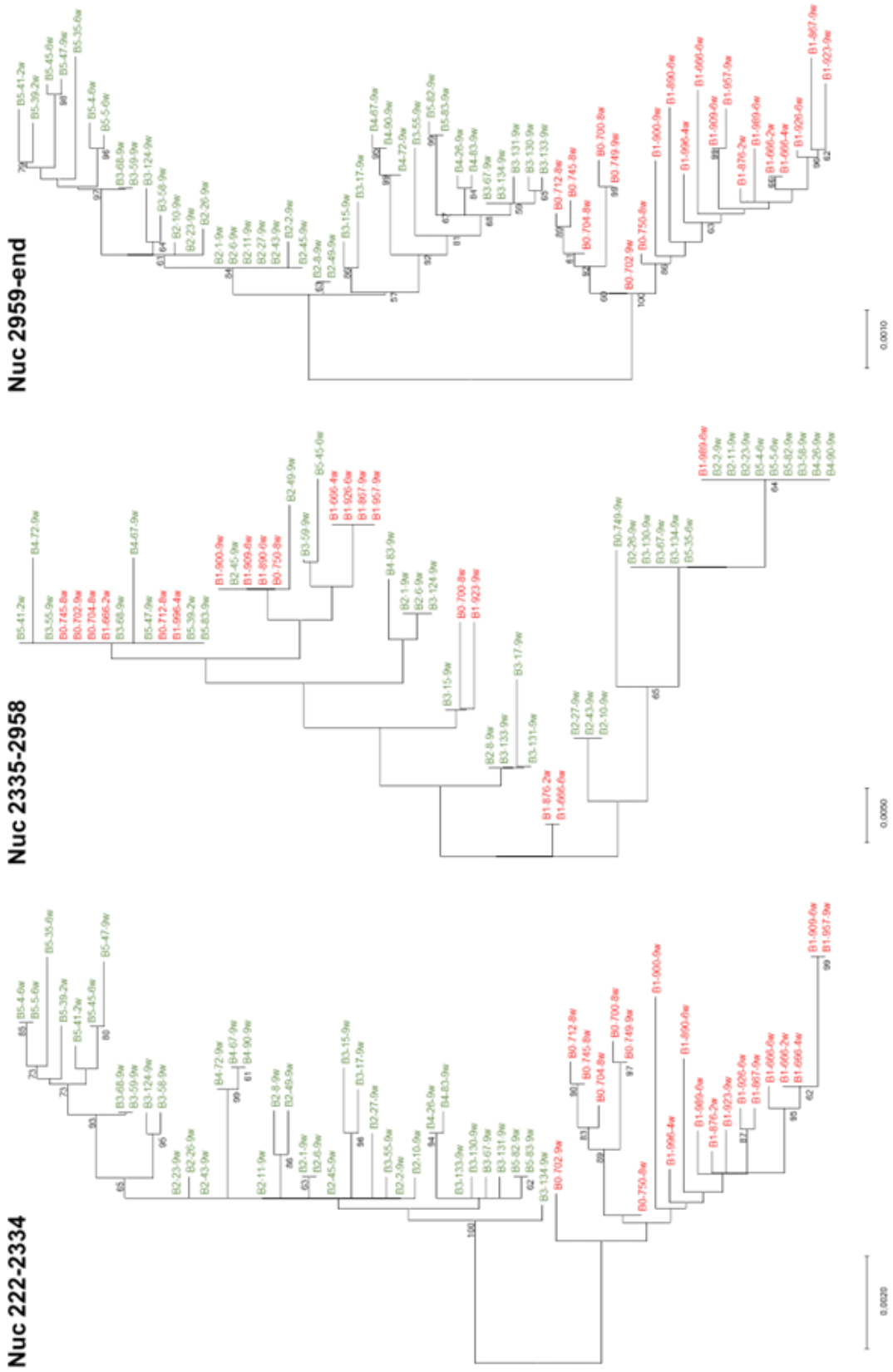
Supplementary Figure 2. Bayesian phylogenetic tree constructed using the whole genome sequences of PRRSV-1. The red and green shaded areas denote the sequences that were obtained from Strain 1 and 2, respectively. Lelystad strain is the prototype for PRRSV-1. The strains that are coloured in red represent the commercially licensed vaccines in Spain. Only posterior probability values >70% are shown.



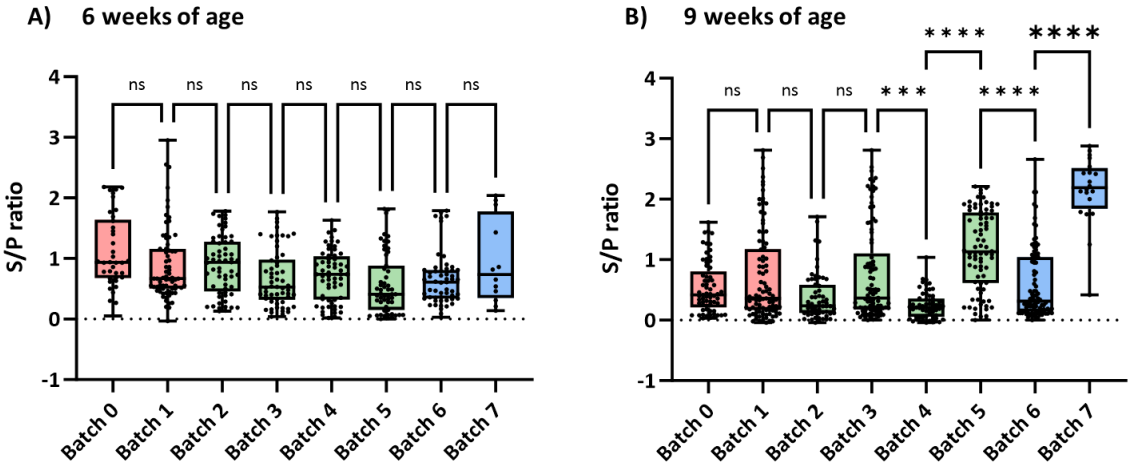
Supplementary Figure 3. Bayesian phylogenetic tree based on ORF5 sequences of PRRSV-1. The red and green shaded areas represent sequences from Strain 1 and 2, respectively. Lelystad strain is the prototype for PRRSV-1. The red-coloured strains indicate vaccines licensed in Spain. Only posterior probability values >70% are shown.



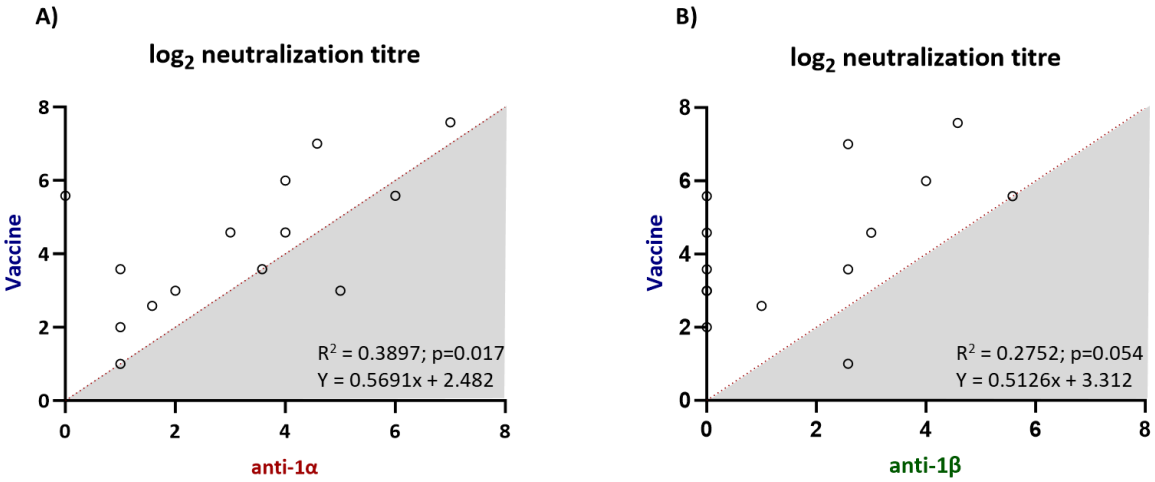
Supplementary Figure 4. Genome recombination event between variants 1α and 1β. Phylogenetic trees constructed based on the resulting fragments from the recombination event in nsp2 (nucleotide positions 2,335-2,958). Isolates of variant 1α are shown in red and of variant 1β in green. Nucleotide position correspond to the alignment with the prototype PRRSV-1 strain Lelystad (LV; NC_043487).



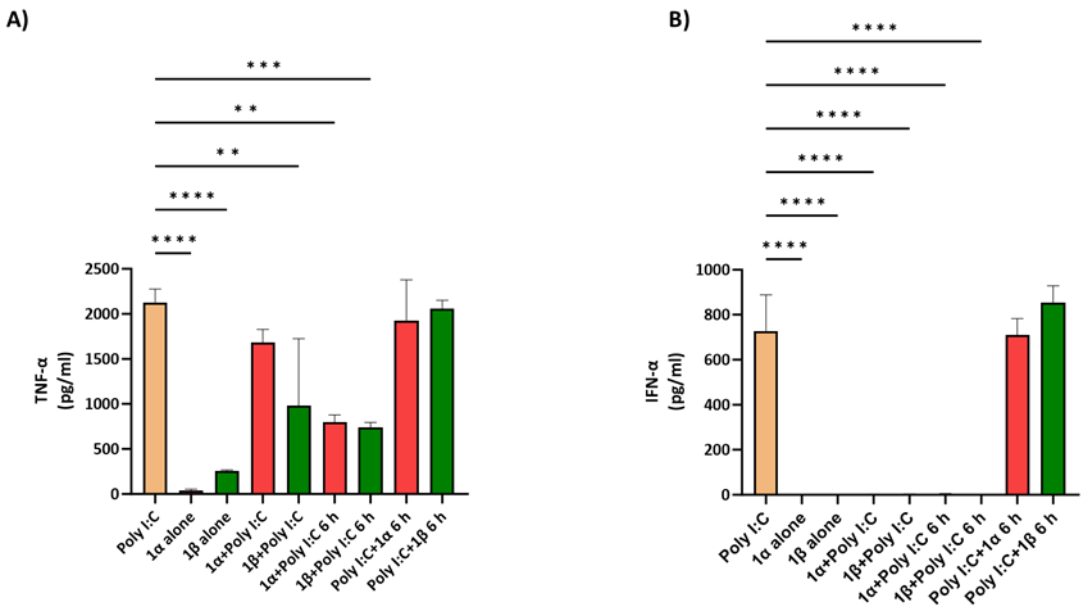
Supplementary Figure 5. Antibody levels of six- and nine-week-old piglets against the viral nucleocapsid protein expressed as S/P ratios as determined in ELISA. Each dot represents an individual. A) Animals examined at six weeks of age; B) Animals examined at nine weeks of age. ns=non-significant differences. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.



Supplementary Figure 6. Neutralizing antibody titres in sows from Batch 1 against the variants 1α and 1β and the vaccine virus. Comparison of neutralization titres (log_2) obtained for each sow from Batch 1 with variants 1α (A) or 1β (B) with the titres against the vaccine used in the farm. The diagonal (dotted red line) represents the line of identity for both tests.



Supplementary Figure 7. Inhibition of the levels of poly I:C induced IFN-α and TNF-α in PAM. The graphs show the levels of IFN-α (A) and TNF-α (B) in the cell supernatants of PAM after being inoculated with the variants 1α and 1β in different treatment combinations with poly I.C. virus+Poly I:C= both simultaneously; virus+Poly I:C 6h= virus followed by poly I:C 6h later; Poly I:C+virus 6h= poly I:C followed by virus 6h later. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.



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Chapter 5.

Study 3

5. STUDY 3

A single recall vaccination lapse in sows triggers PRRSV resurgence and boosts viral genetic diversity.

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5.1. ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) persists on certain farms despite vaccination and control efforts. Although underlying reasons for this phenomenon remain elusive, a suspected contributing factor is its genetic diversity. This study aimed to examine the evolution and persistence dynamics of PRRSV-1 within an endemic and vaccinated farm.

An eight-month observational study was conducted on a farrow-to-fattening farm, housing 1,700 quarterly-PRRSV-vaccinated sows. The study covered three farrowing batches, monitoring piglets from birth to nine weeks of age (RT-qPCR of umbilical cords and sera). An incident related to elevated temperatures during the summer occurred during the last blanket vaccination before sampling the third batch. Whole-genome sequencing (55 samples, Illumina Miseq) and ORF5 Sanger sequencing (213 samples) were performed. Anti-PRRSV ELISA antibodies and neutralising antibody levels against the vaccine strain were assessed at three weeks of age.

Viral circulation was primarily confined to the nurseries, with occasional vertical transmission. A notable surge in circulation was observed in Batch 3, limited to six- and nine-week-old pigs. Remarkably, the percentage of seropositive piglets at three weeks of age in Batch 3 significantly dropped compared to earlier batches, likely due to the vaccination failure during summer. Phylogenetic analyses showed the persistence of the same viral strain throughout the study, with increased genetic diversity in Batch 3. Both selection and recombination contributed to PRRSV diversity in this herd. Ultimately, reestablishing the vaccination program led to a PRRSV-positive-stable with vaccination status.

Overall, a single vaccination lapse caused increased PRRSV-1 incidence and genetic diversity in weaners, linked to declining maternal antibody levels, underscoring the importance of strict vaccination adherence.

5.2. INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) infection causes severe economic losses in affected farms (Holtkamp et al., 2013; Nathues et al., 2017; Renken et al., 2021). Following the introduction of the virus into a breeding herd, the infection usually establishes an endemic cycle (Albina, 1997) where susceptible sows will deliver viraemic-born piglets that will carry the infection into the nurseries. One of the key control measures for PRRSV infection involves implementing a vaccination program in sows to limit vertical transmission, contributing thus to herd stabilization. Once the stabilization is achieved, eliminating the infection from nurseries may often require partial depopulation (Corzo et al., 2010). However, some herds may experience new outbreaks or rebounds in PRRSV incidence due to the resurgence of the same viral strain. Various factors may contribute to these re-emergences of the resident PRRSV strain, being the presence of non-immune breeders one of the main circumstances. This study investigates the impact of a single instance of non-compliance with recall vaccination in the breeding herd on the resurgence of PRRSV infection and viral genetic diversity.

5.3. METHODS

5.3.1 Case farm and follow-up chronology.

A 1,700-sow farrow-to-fattening farm was being monitored in the frame of a viral evolution study. The farm operated on a three-week farrowing batch schedule, with approximately 240 sows per batch. Replacement gilts were purchased from a PRRSV-negative farm at 5.5 months of age, and subsequently introduced into the breeding herd following a six-week quarantine. The PRRSV vaccination plan included two doses of a modified live vaccine (PYRSVAC-183, SYVA Laboratories) during the acclimatization period, along with quarterly recall vaccinations to the breeders with the same vaccine.

The ongoing viral evolution study aimed to examine the evolution of the circulating PRRSV from birth to the end of the nursery phase in endemic farms. To accomplish this, animals from three farrowing batches (designated as batches 1-3) were monitored over an eight-month period. One of the recall PRRSV vaccinations of the sows was scheduled six weeks before the expected farrowing date of Batch 3; namely, before day 70 of gestation.

This vaccination took place in late July, coinciding with maximum temperatures exceeding 35°C. Ventilation in the gestation barns was operated manually. During the vaccination process, the reconstituted vaccine was left at room temperature for several hours. Although a precise record of the internal barn temperatures is unavailable, the room temperature exceeded the comfort range for the sows and the recommended conditions for conserving the reconstituted vaccine (25°C).

5.3.2. Sampling and PRRSV detection.

In the three examined farrowing batches, a total of 535 piglets from 151 sows were followed from birth to nine weeks of age. Samples were collected at birth (umbilical cords, UC), with subsequent blood collection from the piglets at three, six, and nine weeks of age. UC were processed following a previously described protocol (Martín-Valls et al., 2018) and blood samples underwent centrifugation at 300 x g for five minutes before storage at -80°C.

Viral RNA extraction was conducted using MagMax Core Nucleic Acid Purification 264 Kit (Applied Biosystems, Thermo Fisher Scientific, United States), following the manufacturer's instructions. The PRRSV status was determined using a commercial RT-qPCR kit (VetMAX™ PRRSV EU & NA 2.0 Kit, Thermo Fisher Scientific), including an internal positive control in each sample. Samples yielding C_t values <37 were considered positive.

Vertical transmission was determined by identifying cases where at least one positive UC per litter was detected by RT-qPCR. Cumulative incidence rates were calculated by dividing the number of new cases by the number of susceptible animals through each observation period. Calculations excluded animals for which data was incomplete or unavailable.

5.3.3. Serological analyses.

Anti-PRRSV antibodies were determined in all serum samples of three-week-old piglets through a commercial ELISA kit (IDEXX PRRS X3 Ab Test, IDEXX, United States). Additionally, the level of neutralising antibodies was determined in 50 three-week-

old piglets per batch matched by parity of the sow and randomly selected from those testing negative by RT-qPCR up to that age. The VNT was conducted using the vaccine strain used on the farm according to Yoon et al. (1994) with minor modifications.

5.3.4. Sequencing and phylogenetic analyses.

Samples with C_t values <32 (all in Batch 1 and 2 and 50% in Batch 3) were ORF5 Sanger sequenced, following a previously described protocol with minor modifications (Mateu et al., 2003). Viral isolation in porcine alveolar macrophages (PAM) was conducted for at least 20% of the sequenced samples, and the resulting cell-culture supernatants underwent whole-genome sequencing (WGS) using Illumina MiSeq RNAseq, in accordance with a previously detailed protocol (Clilverd et al., 2023).

The consensus sequences obtained from both whole genome (n=55) and the ORF5 (n=213) sequencing were submitted to GenBank with the Accession Numbers PP261834 to PP261888, and PP261642 to PP261833, respectively.

A subset of sequence analyses was performed including: the construction of phylogenetic trees through Bayesian inference using Mr. Bayes (Huelsenbeck and Ronquist, 2001, available at https://ngphylogeny.fr); the determination of nucleotide identities within and between clades with p-distance using MEGA XI (Tamura et al., 2021); the prediction of N-glycosylations in the viral glycoproteins using Net-N-Glyc 1.0 (Gupta and Brunak, 2002, available at https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/); and the evaluation of recombination patterns using GARD algorithm method (Pond et al., 2006). In addition, the amino acid composition of the predicted sequences was compared between the identified clades.

5.3.5. Statistical analyses.

PRRSV incidences were compared using the $\chi 2$ test (Fisher's exact test). Comparison of S/P ratios and levels of neutralising antibodies were performed using the Kruskal-Wallis test. All statistical analyses were conducted using GraphPad Prism v10, with significance set at $p<0.05$.

5.4. RESULTS

At the beginning of the study, in Batch 1, viral circulation was primarily restricted to the nurseries, with sporadic occurrences of vertical transmission (Table 1). In the following batch, Batch 2, the incidences at farrowing units and nurseries decreased. A significant rise in PRRSV circulation was observed in the third batch, mainly affecting six- and nine-week-old pigs (from 6.1% to 98.8% incidence at six weeks of age in Batch 2 and 3), while vertical transmission remained unaffected.

Table 1. Data on the monitored animals, vertical transmission frequency, and cumulative incidences at three, six, and nine weeks of age for each followed batch.

Batch	Nº litters	Range of sows' parities	Pigs followed (1-9 woa¶)	Litters with PRRSV+ piglets at birth	Incidence at 3 woa	Incidence at 6 woa	Incidence at 9 woa
1	44	1-9	170	1 (2.3%)[a] ($CI_{95\%}$: 0.1-13.5%)	6.5%[a]	19.9%[b]	60.8%[b]
2	42	1-9	173	3 (7.1%)[a] ($CI_{95\%}$: 1.9-20.6%)	4.1%[a]	6.1%[c]	20.9%[c]
3	65	1-8	192	3 (4.6%)[a] ($CI_{95\%}$: 1.2-13.6%)	7.3%[a]	98.8%[a]	100.0%[a]
Totals/ Average	**151**	**1-9**	**535**	**4.6%**	**N.A.**	**N.A.**	**N.A.**

¶ woa = weeks of age. N.A. does not apply. Values with a different superscript letter indicate significant differences ($p<0.05$)

In Batch 3, the proportion of seropositive piglets at three weeks of age dropped significantly compared to earlier batches (83% and 88% in batches 1 and 2 vs. 37% in Batch 3) (Figure 1), regardless the sow's parity (data not shown). Interestingly, a group of fourteen sows were sampled in both batches 1 and 3. The average S/P ratios of their piglets at three weeks of age were significantly different between batches (1.03±0.08 vs. 0.44±0.35, respectively; $p<0.0001$), indicating a decrease in the levels of PRRSV antibodies in Batch 3 compared to Batch 1. When evaluating the neutralising antibodies titres in fifty randomly selected three-week-old piglets with no prior infection up to that age, the results (Figure 2) showed that median neutralisation titres against the vaccine virus ranged from 5-6 $\log_2$ in batches 1 and 2, whereas the median titre for Batch 3 piglets was 2.0 $\log_2$ ($p<0.05$). This decrease could be attributed to the vaccination failure during the summer months.

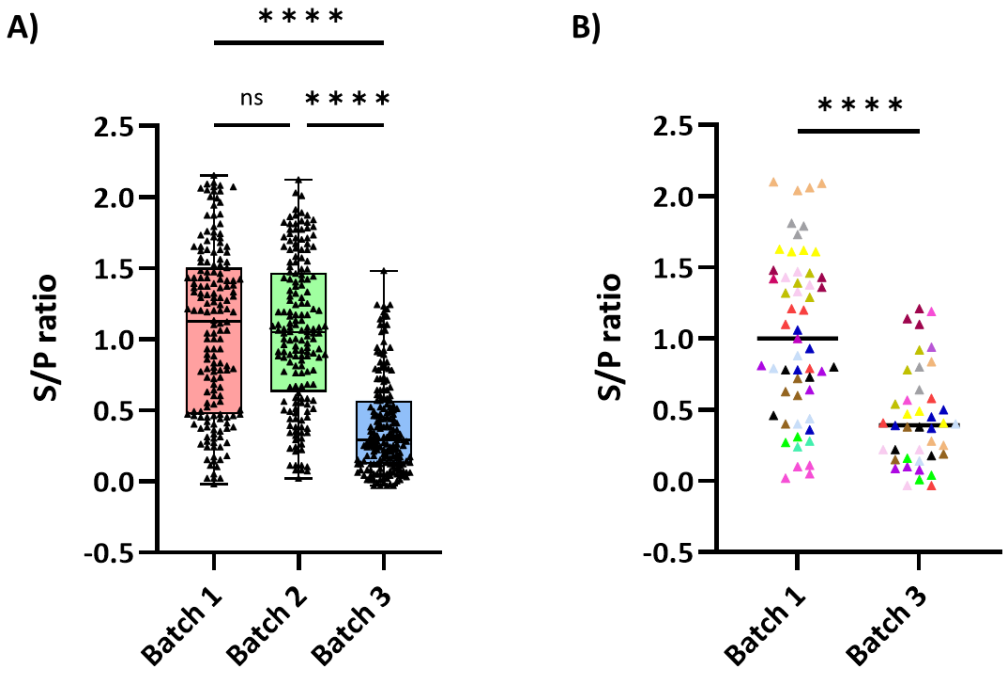


Figure 1. PRRSV-antibody levels of three-week-old piglets as determined by ELISA (S/P ratios). Each triangle represents an individual. S/P ratios ≥0.4 are considered positive. A) Distribution of S/P ratios for all examined animals per batch. B) Distribution of S/P ratios for the offspring of the fourteen sows present in batches 1 and 3. Offspring from the same sow is depicted using the same colour. ns= non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

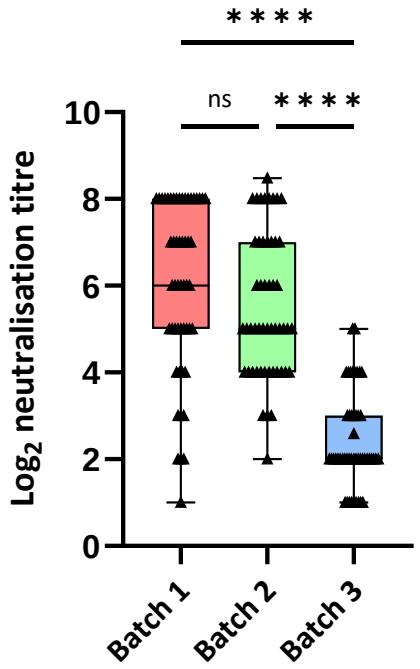
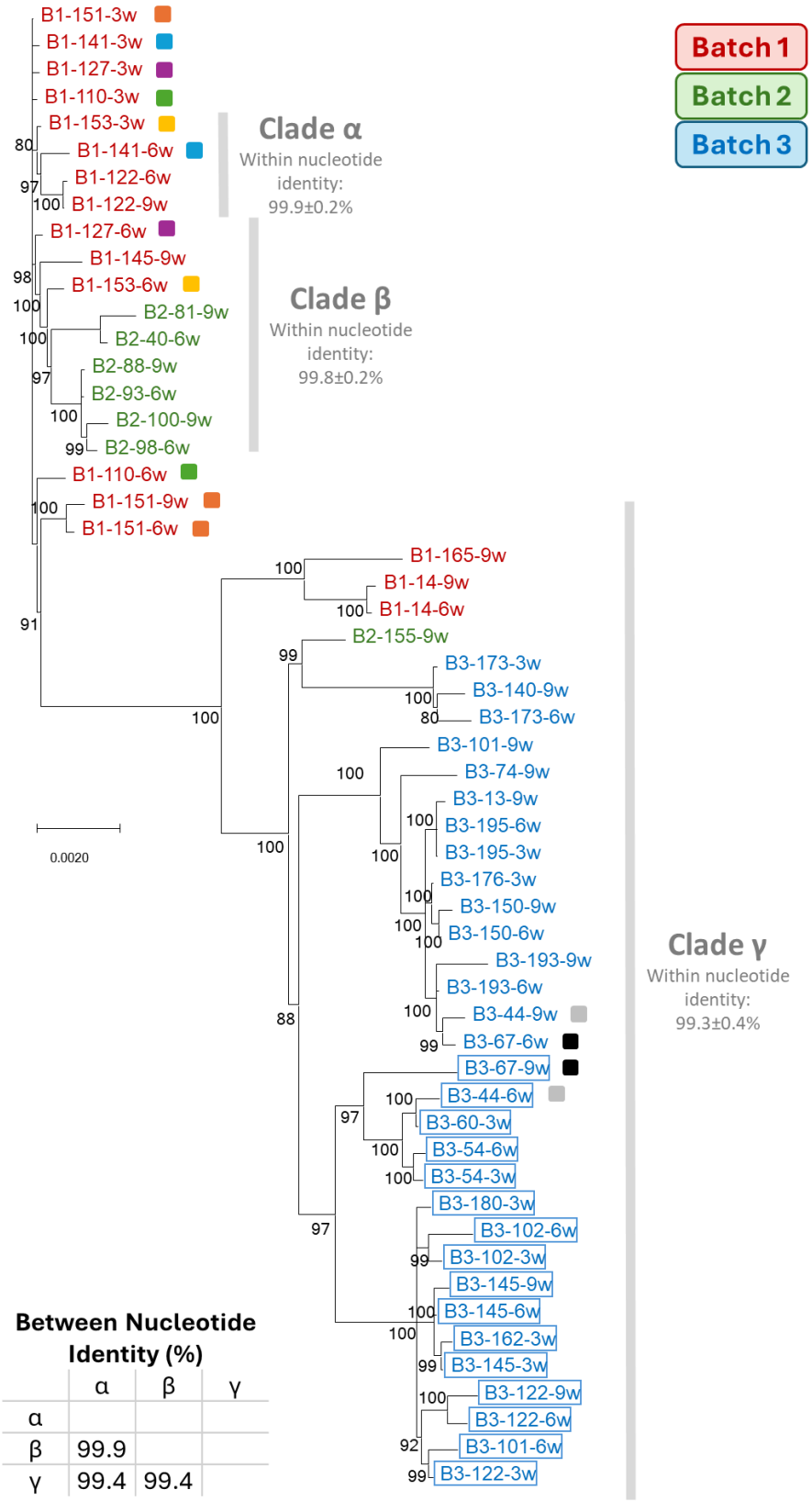


Figure 2. PRRSV-neutralising-antibody titres (log_2) of three-week old piglets as determined by viral neutralisation test. Each triangle represents an individual. ns= non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Phylogenetic analyses based on the complete genome (Figure 3) and ORF5 (Supplementary Figure 1) revealed the consistent presence of the same viral strain throughout the study period, identifying distinct circulating clades. The overall nucleotide identity for the complete viral genomes retrieved from all three batches was 99.2% ± 0.04%. None of the sequences corresponded to the vaccine used on the farm (86.2% nucleotide identity). In Batch 1, at least three circulating clades were distinguished, designated α, β, and γ. Clade α was not further detected in subsequent batches. For Batch 2, all animals harboured clade β, except one, infected by γ. In Batch 3, the γ clade prevailed, being the only one detected. Interestingly, Batch 3 exhibited higher within-genetic diversity compared to previous batches (0.54% ± 0.04% vs. 0.37% ± 0.01% and 0.39% ± 0.03% in batches 3, 1, and 2, respectively), coinciding with the sharp increase in PRRSV incidence at six weeks of age. Notably, in Batch 3, mortality in the nurseries rose to 15% from the previous average of less than 2%.

Clade γ differed from the other clades at 83 amino acid positions throughout the genome, of which 36 corresponded to fixed amino acid substitutions, and the remaining 47 presented variable proportions of the previous amino acid and a new one. These amino acid substitutions were not randomly distributed across the genome. The 36 fixed amino acid substitutions accumulated mostly in nsp2 (11), nsp10 (4), GP3 (4), and M protein (4), representing a 1.1%, 0.9%, 2.3%, and 1.5% of each protein, respectively, and were mostly present since the first γ isolates in Batch 1 (25/36) and Batch 2 (8/36). The 47 variable amino acids sites identified in clade γ appeared mostly in Batch 3 (41/47) and accumulated mainly in nsp2 (20), nsp5 (4), nsp1b (4), and nsp3 (3) (2.0%, 2.4%, 1.6%, and 1.0% of each protein, respectively). Clade γ had an additional potential N-glycosylation site in GP3 at position 27 compared to the amino acid sequence of Lelystad virus (LV, Genbank accession number NC_043487).

It is worth noting that sixteen isolates in clade γ harboured a truncated GP3 protein resulting from a stop codon (position 241 corresponding to LV). Truncation was confirmed by ORF3 Sanger sequencing. Sequences with this truncation clustered together within the γ clade. Moreover, isolates from all batches harboured an amino acid deletion in GP3 at position 246 and in GP4 at position 66, corresponding to LV, both located in described neutralising epitopes (Costers et al., 2010; Vanhee et al., 2011).



Between Nucleotide Identity (%)

	α	β	γ
α			
β	99.9		
γ	99.4	99.4	

Figure 3. Bayesian analysis of the whole genome sequences obtained in this study. Posterior probabilities higher than 70% are shown. The colour-coded representation designates sequences retrieved from animals of Batches 1, 2, and 3 in red, green, and blue, respectively. Individuals whose isolates from different ages clustered into a different clade are indicated with the same-coloured square. Blue-coloured boxes indicate isolates harbouring a truncated GP3.

Moreover, the analyses revealed that, in seven animals, isolates obtained at different ages from the same individual grouped into distinct clades. Recombination analyses suggested that the differences in clustering observed in four of these cases (sequences B1-141, B1-151; B1-153 and B3-67 in Figure 3) might potentially result from genetic recombination between different clades, while in one case (B3-44), it could be attributed to a reinfection from a different clade. The distinct classification for the remaining two individuals (B1-110; B1-127) could not be definitively determined (Supplementary Figure 2).

Noteworthy, the reestablishment of the vaccination program eventually led the farm to achieve a PRRSV-positive-stable with vaccination status one year after Batch 3 (data not shown).

5.5. DISCUSSION

Before the vaccination issue arose, the PRRS control program in the farm was limiting the spread of the infection at early ages. Despite vertical transmission was detected in 2-7% of litters, horizontal transmission in nurseries remained restricted and the reproductive performance was acceptable. This limitation in the transmission was probably attributable to the maternally-derived immunity together with proper management practices in nurseries.

The fortuitous coincidence of the vaccination issue with one of the batches under observation provided the opportunity to examine on field the impact of unproper recall vaccination on the incidence and the genetic diversity of PRRSV. As evidenced by the serological results, the failure of just one recall vaccination resulted in a dramatic drop of maternally-derived antibodies in piglets. This happened for all sows, regardless of the parity, indicating that the decline of antibody titres was not completely palliated by receiving more or fewer vaccine doses in the past. This decline in the antibody titres probably facilitated an earlier and wider spread of the virus in nurseries, with almost all piglets becoming infected between three and six weeks of age. Since no other changes were recorded in the management of the farm, the unproper vaccine handling stands out as the most likely explanation. Another contributing factor might be the thermal stress experienced by the sows during that period, arising from the elevated temperatures, which

could compromise their immune response development after vaccination. Interestingly, the proportion of sows delivering viraemic animals was not affected. This most probably suggests that protection against vertical transmission relies more on the cell-mediated immunity rather than on the neutralising antibody response raised by vaccination.

Additionally, this increased transmission was coupled with an increase in the genetic diversity of the virus. This phenomenon aligns with the larger number of infected animals at early ages, with almost all pigs in Batch 3 being infected at six weeks of age, contributing to the subsequent expansion of the viral cloud. This fact underscores the importance of a strict adherence to vaccination schedules in endemic herds. While it can be argued that infected animals will eventually reach nurseries, and sooner or later the virus will spread, field evidence suggests that the earlier the age of infection, the greater the impact of PRRSV on the farm. In our case, this earlier circulation resulted in a sharp increase of the mortality in nurseries that rose up to 15% in the third batch from less than 2% in batches 1 and 2.

Furthermore, despite the initial circulation of at least three viral clades (α, β, and γ) in this herd, only two clades circulated in the second batch (β and γ), of which only one endured into the third batch, where it underwent further diversification (γ). This suggests the influence of selection as the evolutionary force that favoured a specific viral clade while extinguishing others, both from Batch 1 to 2 and Batch 2 to 3. Additionally, upon the reintroduction of the vaccination program, the newly dominant clade faced extinction. Hence, not all variants possess equal capabilities for long-term persistence on farms, and despite their predominance at a given moment, they may vanish when conditions change. Investigating the reasons behind the persistence or disappearance of specific variants could provide valuable epidemiological insights. In our case, passive immunity received by piglets was probably one of the key drivers of the viral evolution.

One notable observation is the identification of a subclade of the γ clade harbouring a truncated GP3 on the 3’ end. The predicted amino acid sequence in this case would be 241 amino acid long. GP3, in conjunction with GP2 and GP4, forms a heterotrimer that interacts with CD163(Das et al., 2010; van Gorp et al., 2009), the essential viral receptor in porcine alveolar macrophages (Calvert et al., 2007). This finding suggests that the deleted segment would not be essential for such interaction and agrees with findings from previous reports (Forsberg et al., 2001; Frossard et al., 2013; Ropp et al., 2004; Wang et al., 2019).

Moreover, among the seven instances where isolates from different timepoints in the same individual formed separate clusters, four were likely the result of recombination. This underscores a notable frequency of interclade recombination events. The occurrence of recombination appears to be more widespread than previously thought, possibly owing to the limitations of previous sequencing methodologies. These limitations involved both only identifying those recombination events within the sequenced segments of the genome and only detecting those recombination variants that became predominant. WGS with current methodologies has brought to light a higher frequency of recombination events that were previously overlooked.

5.6. CONCLUSIONS

These findings emphasize the critical importance of strictly adhering to vaccination schedules and implementing robust vaccination procedures to exert an effective impact on PRRSV viral circulation. Within this herd, the drop in the humoral immunity, the enhanced transmission and recombination emerged as substantial driving forces contributing to the genetic diversity of PRRSV.

Ethics approval and consent to participate.

The animal study was reviewed and approved by Ethics Committee in Animal and Human Research of the Universitat Autònoma de Barcelona (approval numbers 3221-CEEA-UAB and CEEAH-5691 for the project and the procedure, respectively).

Availability of data and materials.

The datasets of sequences are available GenBank and accession numbers can be found in the main text.

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Author's contributions.

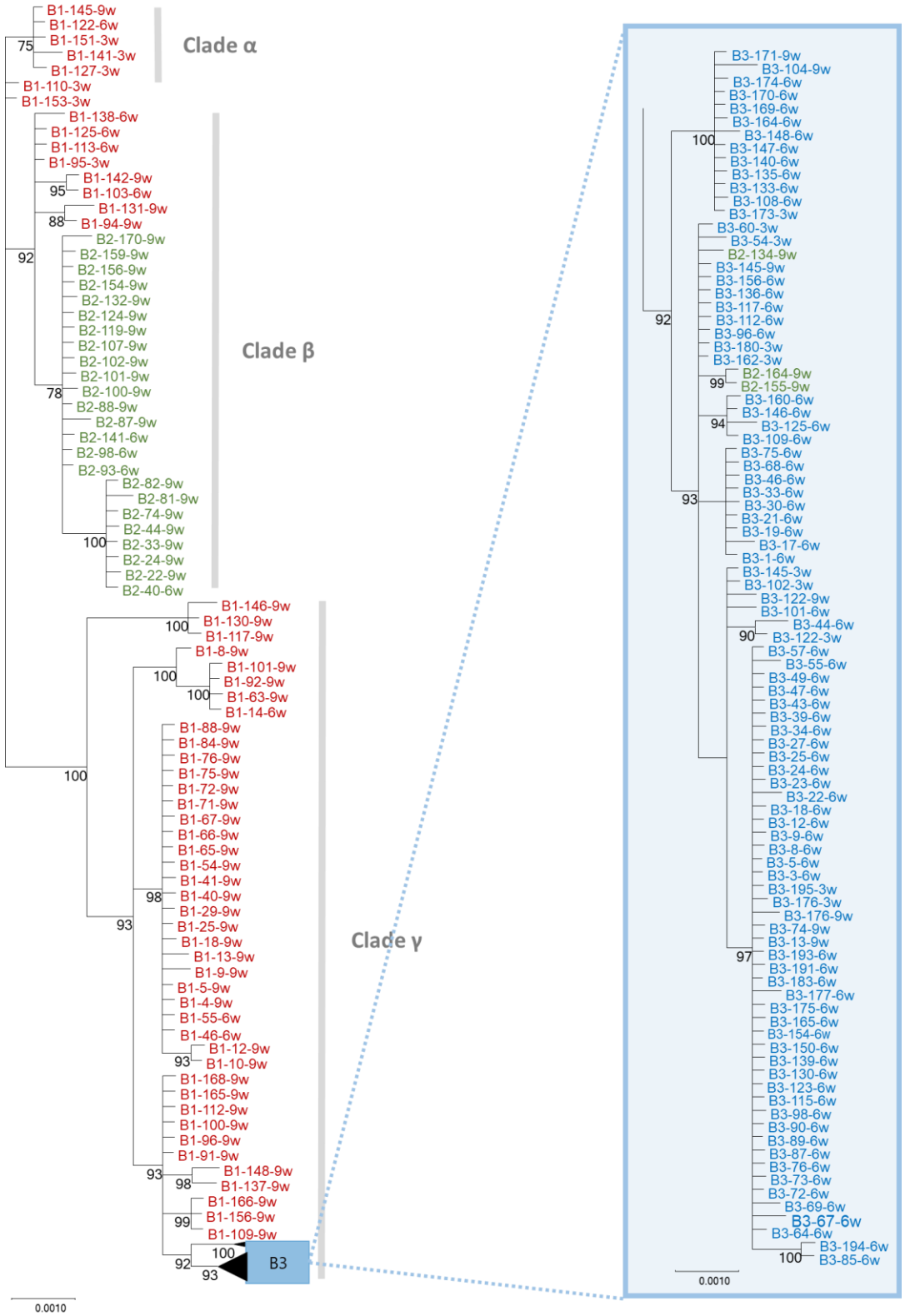
Hepzibar Clilverd: Investigation, Formal analysis, Visualization; Writing - Original Draft. Gerard Martín-Valls: Methodology, Investigation, Project administration, Writing – Review and Editing. Yanli Li: Investigation, Writing – Review and Editing. Marga Martín: Conceptualization, Methodology, Writing – Review and Editing. Martí Cortey: Conceptualization, Methodology, Software, Formal analysis, Writing – Original Draft, Supervision. Enric Mateu: Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Supervision.

Declaration of competing interest

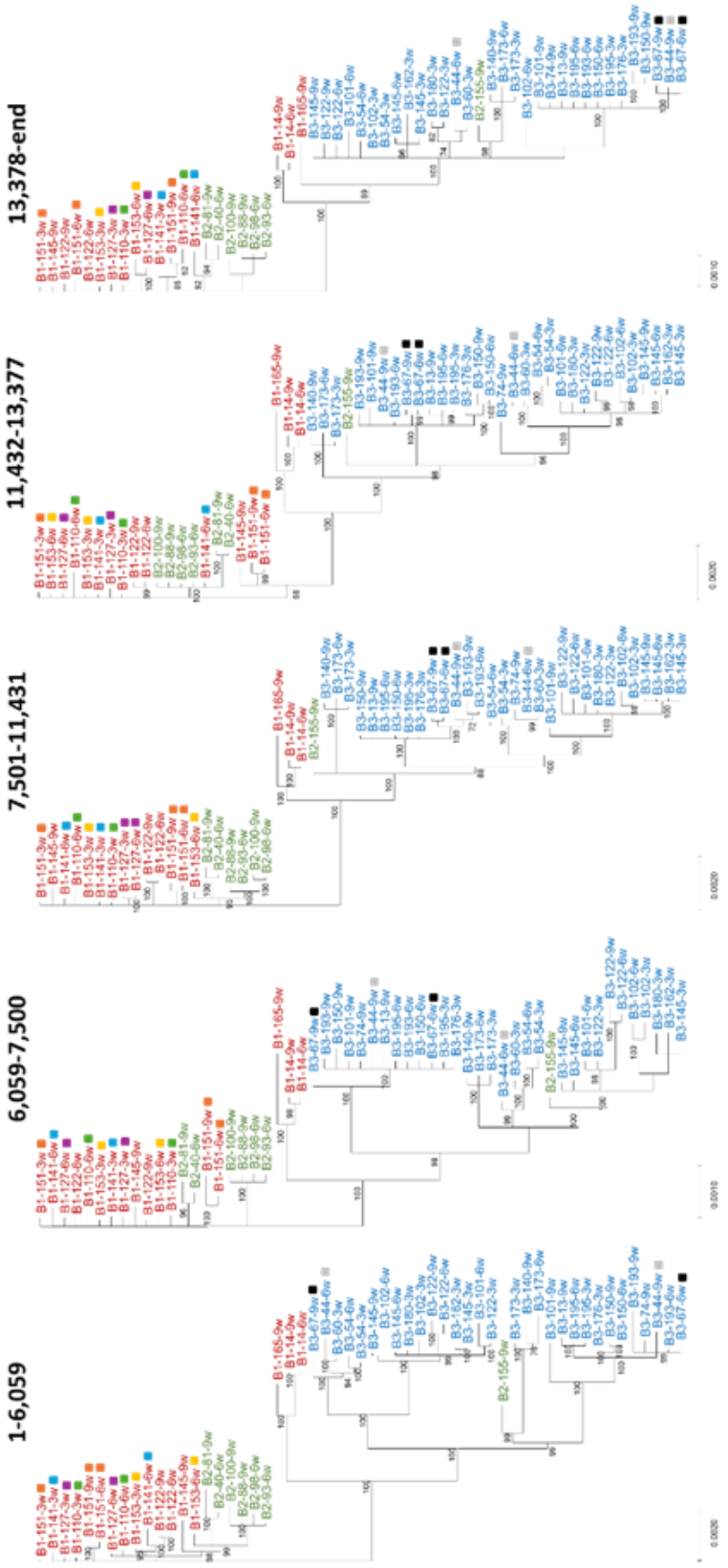
The authors declare no competing interests.

5.7. SUPPLEMENTARY MATERIAL

Supplementary Figure 1. Bayesian analysis of the ORF5 sequences obtained in this study. Posterior probabilities higher than 70% are shown. The colour-coded representation designates animals from Batches 1, 2, and 3 in red, green, and blue, respectively. Additionally, the identified clades from the complete genome analyses have been marked.



Supplementary Figure 2. Recombination analysis of the whole genome sequences obtained in this study. Phylogenetic trees were constructed based on each recombinant fragment, incorporating the whole genome sequences of Batch 1 (in red), 2 (in green), and 3 (in blue). Individuals whose isolates from different ages clustered into a different clade are indicated with the same-coloured square.



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Chapter 6.

General discussion

6. GENERAL DISCUSSION

The PhD dissertation delves into the dynamics and evolution of PRRSV within endemically infected farms with persistent circulation of the virus. It sheds light on three distinct scenarios: a) the post-outbreak period, illustrating the establishment of the endemic situation following the introduction of PRRSV into the farm, b) a persistent circulation within a farm that, despite implementing rigorous control measures, has been unsuccessful in eliminating the virus and, c) a positive-unstable herd with low prevalence that was implementing a vaccination program.

One of the key strengths of this study lies in its real-time observation of how these events unfold under field conditions, providing insight into how the virus can adapt to the changing conditions within the herd. The results show how the viral diversity is created, giving rise to variants fit to prevail under different conditions, and how selection acts, along with some of the driving forces behind this selection. Also, the study reveals the abrupt nature of the replacement of one variant by another or by another strain, challenging the notion of a gradual transition or a long coexistence between variants. Moreover, it unveils the diverse contribution of different animals to viral transmission, including the existence of superspreaders. What was once theoretical has now been substantiated through the observation of these occurrences within a farm. Certainly, we did not observe the full spectrum of potential outcomes of PRRSV infection in a herd, but we presented some examples of what can actually happen.

Initially, our hypothesis presumed that most of the observed genetic diversity of the virus in the farm would likely be generated by the infection of a large number of animals in nurseries or fattening units. The rationale behind this was simple: the greater the number of infected animals, the larger the viral cloud and, consequently, the higher the chances for new variants to arise. This was observed after the vaccination issue in Study 3; however, this proved to be a naïve view of the problem.

The present dissertation, as presented in Study 1, highlighted that transplacental transmission, with its associated bottlenecks, may be a major determinant of the genetic diversity within the farm - particularly in the first weeks following the introduction of a new strain. At present, though, the specific bottlenecks or factors involved in this vertical transmission that led to the generation of the observed diversity have not been identified

yet. However, it seems improbable that this phenomenon is entirely random; otherwise, a new variant would emerge in each vertical transmission event, which is not the observed case. Furthermore, the findings from Study 2 showed that the introduction of strain 2 did not result in higher virus diversity than strain 1, pointing towards the involvement of additional influencing factors.

An important question arises here: What role does immunity play in the generation of new variants during vertical transmission? Results from Studies 1 and 2 indicated that immunity plays a crucial role in the selection of escape variants, but understanding what happens in transplacental infection is more complex. First, the overall comprehension of what transpires in sows with only partial heterologous immunity, such as through vaccination, remains elusive. For vertical transmission to occur, the virus must reach first the placenta, which can only happen during a viraemic phase. How do heterologous neutralizing antibodies selectively determine which viral variants reach the placenta? Is cell-mediated immunity in the maternal-foetal interphase exerting additional selection pressure on variants crossing to the foetus? Moreover, how does the virus cross the placenta - via maternal macrophages reaching a foetus or as free virions transported across the tissues? Is this adding additional bottlenecks? At present, these questions remain unanswered, but results of Study 3 gave us some hints.

The fortuitous vaccination incident revealed that a single failure to administer a recall vaccine dose resulted in a drop of the maternally-derived antibody levels in piglets, coupled with a rise in the incidence of the infection between three and six weeks of age. However, vertical transmission rates were not affected, pointing towards the cell-mediated immunity as the force controlling transplacental infections.

In Study 1, we also observed that the extensive genetic diversity generated immediately after the introduction of the new strain was substantially reduced when a specific variant was selected to become predominant. Despite the initial burst of viral diversity, only a few variants were capable of prevailing. In Farm 1, evidence suggested that five months after the initial outbreak, a large proportion of sows and pigs had been infected and subsequently developed immunity. RT-qPCR and the serological analyses at that time indicated that incidence was very low in the farrowing units and nurseries, with declining maternal antibody levels, reinforcing the notion of low viral circulation. Following this period, only one viral variant could be found in the farm. The most

plausible explanation is that it was the only variant capable to persist transmitting within a herd where most adult animals or piglets had some level of immunity, whether active or passive.

If the first study took place a few weeks after the outbreak, the second study presented a completely different scenario involving a herd where PRRSV had been circulating for several years. The evolution of the virus before the study was unknown, but there were no evident determinants for persistence, such as poor vaccination practices or inadequate gilt and herd flow management. While the frequency of lateral introductions remained uncertain, the external biosecurity measures could be categorized as average.

Upon examining the molecular epidemiology of the infection, two major evolutionary events became apparent during the observation period: the emergence of a variant of the resident strain and, later, the introduction of a new strain. The emerging variant was characterized by being a neutralization escape mutant with an enhanced affinity for attaching to macrophages. Intriguingly, the replacement of the original variant by the emerging one was abrupt. Evidence indicated that the twenty-five synapomorphic mutations identifying the variant did not accumulate gradually, denoting it was not a product of drift. While most of these mutations were present in the quasi-species before detecting the variant, their frequencies in the quasi-species were diverse and not linked one to another. For instance, certain nsp1 mutations could be as frequent as 16%, while mutations in GP2 in the same animal could be five to eight times less frequent. It seems unlikely that the virus underwent accelerated selection of beneficial mutations in GP2 unlinked to those in nsp1 within a few weeks.

A simpler explanation could involve a recombination event, where a viral genome harbouring favourable mutations in one region recombined with another genome carrying favourable mutations in a different region. The result of such recombination would be synergistic, producing a fitter variant with improved attachment to macrophages and enhanced ability to escape neutralization. Once the new variant emerged, its superior attachment to macrophages led to increased replication, which, in turn, facilitated transmission, resulting in the rapid replacement of the old variant. Supporting this hypothesis is the evidence of recombination between variants 1α and 1β. Moreover, this phenomenon mirrors the swift shifts in viral variants observed with SARS-CoV2[1050,1051].

Collectively, these results underscore the remarkable plasticity of PRRSV, allowing it to adapt to almost any circumstance as long as transmission persists.

In Study 3, beyond the impact of the vaccination issue, we could also observe how both selection and recombination shaped the viral population. In Batch 1, three viral clades were detected. However, one clade was purged in Batch 2, and upon examination of Batch 3, only one clade was found to be circulating in the population. Once again, selection favoured the fittest variant over time. Interestingly, the recombination analysis showed that some animals had been infected by viruses from two different clades, resulting in the generation of recombinant isolates. These findings provide further evidence supporting the notion that recombination is probably more common than previously thought.

The combined findings from Studies 1, 2, and 3 also provide valuable insights into the transmission dynamics of the virus. Study 1 highlighted a notable variability in the contributions of PRRSV-infected animals to the overall virus transmission, identifying both super-spreader animals and those with no detectable transmission. Moreover, in Study 1, a born-viraemic animal that remained viraemic throughout the entire study did not transmit the infection to any other pig, or at least this was not detected. The existence of super-spreaders has been described in other infectious diseases, such as MERS and SARS-CoV2[1052–1055]. In fact, it has been long postulated that transmission follows the 80/20 law, namely 80% of the transmission is caused by 20% of the infected individuals[1056]. However, the determinants underlying the superspreading phenomenon remain unclear. Some of the factors related to the host that may influence transmission dynamics are immunity, duration of contact with the infectious host, route of infection, and genetic susceptibility, among others[1054].

In both Study 1 and 2, certain sows, despite being immunized through regular vaccinations or prior infections, displayed vertical transmission events of PRRSV. The presence of these sows with vertical transmission seems to be pivotal in perpetuating farm infections and, at least post-outbreak, in contributing to the genetic diversity within the farm. The question arises: Why do sows, even when repeatedly vaccinated, still suffer vertical transmission events? A simple explanation is that their immunity is not strong enough to provide complete protection. Are these sows simply poor responders? A deeper look into our data revealed that some sows can be seronegative even after being repeatedly vaccinated. For example, we identified a parity 11 sow that was seronegative after more

than four years of quarterly vaccination, yet continued delivering viraemic piglets. The existence of non-responder sows has been documented previously[610,719,883,884]. Which role do these non-responder sows play in perpetuating vertical transmission within the herd and contributing to the generation of diversity? A certain fact is that their offspring will lack adequate protection from maternally-derived antibodies, increasing the risk of becoming infected at earlier ages. Long-term monitoring of endemically infected farms, identifying and characterizing sows with vertical transmissions, both from the immunological and genetic perspectives, could provide valuable insights into the role of these sows in the dynamics of PRRSV.

In Study 1, the decline in maternally-derived antibodies months after the clinical outbreak suggested a gradual waning of immunity, indicating a short-lived immunity. This notion was further reinforced by the observations in Study 3, where a single failure of a recall vaccination resulted in a dramatic drop in maternally-derived antibody levels.

This study also holds practical implications for PRRS control. In Study 1 and 2, reinforcing external biosecurity measures could have potentially blocked lateral introductions of the virus. Although the precise reduction in risk achievable through enhanced biosecurity remains uncertain, it is reasonable to assume that strengthening biosecurity measures would be beneficial. However, when considering the emergence of an escape variant, it is challenging to foresee the precise impact of internal biosecurity measures. Furthermore, in the case of contemplating a vaccine change, such a change would not guarantee improved efficacy, as by now this cannot be predicted. However, one fact becomes clear: Ceasing the vaccination program comes with a warning – be prepared for a resurgence of the virus.

Our results also reveal that no other phenomenon than viral evolution is necessary to explain PRRSV's persistence in the population where the virus circulates. In any scenario of viral circulation, even very close to stability, it becomes a matter of probability for the virus to find a gap to breach through and persist circulating within the farm. While certain farms may successfully eliminate the infection after implementing control programs, a percentage of farms may elude eradication simply due to the emergence of new viral variants. This phenomenon provides insights into situations where, despite herd closure and testing negative for PRRSV, the infection resurfaces after a few months.

Hence, to effectively control PRRSV and prevent the emergence of escape variants, it is crucial to impede the viral circulation. Achieving elimination of PRRSV proves challenging without measures capable of providing sterile immunity to block this circulation. But also, the rapid and abrupt nature of these changes leaves little room for timely intervention. By the time a new variant is detected, it might have already disseminated within the herd.

A potential solution to control PRRSV lies in rearing PRRSV non-susceptible pigs, such as gene-edited PRRSV-resistant pigs with CD163 knock-outs or modifications to the CD163 SRCR5 domain[936]. By precluding transmission, this approach could nullify viral circulation, preventing the virus from persisting on the farm. However, the use of genetically modified pigs opens up many other debates.

From a diagnostic standpoint, Study 2 underscores the importance and benefits of employing whole-genome sequencing over partial sequencing of a genomic segment, such as ORF5. If only ORF5 sequencing would had been conducted in that study, the escape from neutralizing antibody response and increased affinity to macrophages might have been solely attributed to the deletion and glycosylation of GP5. Furthermore, without whole-genome sequencing, the recombination in studies 2 and 3 would have gone undetected. The growing cost-effectiveness of whole-genome sequencing makes it an imperative tool, as it brings clarity to the farm's PRRSV dynamics. This advanced tool provides a more in-depth understanding of PRRSV circulation within the farm, allowing for the implementation of more precise and targeted measures.

In summary, the findings of this PhD dissertation highlight the remarkable plasticity and evolutionary mechanisms that PRRSV employs to maintain its persistence within swine breeding herds. This insight not only enhances our understanding of the virus's adaptable capabilities, but also underscores the critical need for the development of innovative vaccines and strategic control measures. Addressing these aspects is essential to effectively combat the persistent challenges posed by PRRSV in swine farming, contributing to improved disease management and overall herd health.

Chapter 7.

Conclusions

Conclusions

1. In the early post-outbreak period, most PRRSV genetic diversity can be related to vertical transmission events, despite their lower frequency compared to horizontal transmission. This suggests that transplacental infection bottlenecks are stronger than those in horizontal transmission, which accounts for most infections.

2. The post-outbreak viral diversity diminishes over time, with only one variant prevailing on the farm. This indicates that variants less fit in terms of transmission present in the previously exposed sows and their offspring are purged, leaving only the most efficient one to persist.

3. In multiple-vaccinated farms, seronegative sows, including older ones likely exposed to the circulating virus, can be present. Furthermore, some of these sows may undergo repeated infections and produce vertical transmission events. These findings underscore the potential significance of the poor-responder breeders in the maintenance of vertical transmission.

4. Within nurseries, certain pigs may serve as superspreaders, while others do not contribute to the transmission of the virus at all, even if they are born viraemic and remaining so for a long period. This highlights the variability in the individual contributions to PRRSV transmission, emphasizing the importance of studying the determinants that govern such differences.

5. In an endemic vaccinated farm, the replacement of a viral variant by a fitter one may occur swiftly, within just a few weeks. The number of fixed mutations, their frequency in the quasi-species of the previous variant, and evidence of recombination between variants suggest a parsimonious explanation: viral replacement occurs through a combination of mutation, selection, and recombination events, rather than a rapid selection of a specific set of key mutations.

6. The impact on PRRSV incidence was equivalent whether it resulted from the emergence of a fitter variant or the lateral introduction of a new strain. This suggests that the key determinant is the fitness of transmission, rather than the overall genetic identity between the new and the resident strains.

7. Viral variants predominating in a farm may show various advantageous properties, including neutralization escape features or enhanced infectivity for macrophages. These findings underscore the plasticity of PRRSV and its ability to generate variants capable of breaching immune system gaps or overcoming transmission bottlenecks. As long as the virus circulates within the farm, fitter variants will emerge, emphasizing that the only efficient approach for PRRSV control is eradication.

8. Adherence to vaccination schedules is essential to reduce transmission and to limit viral diversity. A single failure in a recall vaccination can boost both viral diversity and incidence.

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Appendix

Appendix

Oral presentations in congresses

1. **Clilverd H**, Li Y, Martín-Valls G, Aguirre L, Cortey M, Mateu E. "Evolución del virus del PRRS en granjas endémicas: cuando todo parece igual, pero es muy diferente" in XXVI Jornadas de Porcino de la UAB y AVPC, Cerdanyola del Vallès, Spain (31/01/24-01/02/24).
2. **Clilverd H**, Martín-Valls G, Li Y, Martín M, Cortey M, Mateu E. "A PRRSV-1 variant with few mutations rapidly replaced the circulating strain with similar effects to a newly introduced strain in an endemically infected and vaccinated farm" in 14th European Symposium of Porcine Health Management (ESPHM 2023), Thessaloniki, Greece (31/05/2023 – 02/06/2023).
3. **Clilverd H**, Martín-Valls G, Li Y, Martín M, Cortey M, Mateu E. "The founder variants transmitted by sows are the main source of PRRSV1 genetic diversity in an unstable infected farm" in 12th International Congress For Veterinary Virology (ESVV2022), Ghent, Belgium (20/09/2022 – 23/09/2022).
4. **Clilverd H**. "Evolución del virus del PRRS en granjas endémicas" in XXIV Jornades de Porcí de la UAB i AVPC, Cerdanyola del Vallès, Spain (15/07/22-17/07/22).
5. **Clilverd H**, Cortey M, Martín M, Martín-Valls G, Mateu E. "Characterization of PRRSV-1 transmission routes in an endemic farm identifies conserved phylogenetic clusters" in North American PRRS Symposium 2019 (NA-PRRS), Chicago, United States (02/11/2019 – 03/11/2019).

Poster presentations in congresses

1. **Clilverd H**, Martín-Valls G, Li Y, Martín M, Cortey M, Mateu E. "The founder variants transmitted by sows are the main source of PRRSV1 genetic diversity in an unstable infected farm" in 12th International Congress For Veterinary Virology (ESVV2022), Ghent, Belgium (20/09/2022 – 23/09/2022).
2. **Clilverd H**, Cortey M, Martín M, Martín-Valls G, Mateu E. "Characterization of PRRSV-1 transmission routes in an endemic farm identifies conserved

phylogenetic clusters" in North American PRRS Symposium 2019 (NA-PRRS), Chicago, United States (02/11/2019 – 03/11/2019).

Contribution to other publications related to PRRSV

1. Martin-Valls GE, Li Y, **Clilverd H**, Soto J, Cortey M, Mateu E. Levels of neutralizing antibodies against resident farm strain or vaccine strain are not indicators of protection against PRRSV-1 vertical transmission under farm conditions. BMC Vet Res. 2023 Oct 20;19(1):217. doi: 10.1186/s12917-023-03785-z.
2. Martín-Valls GE, Mortensen P, **Clilverd H**, Li Y, Cortey M, Sno M, Barna T, Terré M, Guerra N, Mateu E. The use of a whole inactivated PRRS virus vaccine administered in sows and impact on maternally derived immunity and timing of PRRS virus infection in piglets. Vet Rec Open. 2022 Apr 5;9(1):e34. doi: 10.1002/vro2.34.

Contribution to other posters

1. Obregon-Gutierrez P, Cortey M, Martín-Valls GE, **Clilverd H**, Correa-Fiz F, Aragón V, Mateu E. "Changes in the nasal microbiome of piglets infected with a highly virulent PRRSV-1 strain correlate with mortality in piglets" in 14th European Symposium of Porcine Health Management (ESPHM 2023), Thessaloniki, Greece (31/05/2023 – 02/06/2023).
2. Martin-Valls G, Cortey M, Coronado L, Domingo I, **Clilverd H**, Mateu E. "Dynamics of Influenza A virus infection in endemically infected pig farms of Spain" in 14th European Symposium of Porcine Health Management (ESPHM 2023), Thessaloniki, Greece (31/05/2023 – 02/06/2023).