PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS IN AUSTRIA – CHARACTERIZATION OF CURRENT FIELD STRAINS

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DECLARATION

I herewith confirm that I have written this PhD thesis independently and I have followed the rules of good scientific practice in all aspects.
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1. Summary / Zusammenfassung

1.1 Summary

Since more than 25 years, *Porcine reproductive and respiratory syndrome virus* (PRRSV, family *Arteriviridae*, order *Nidovirales*) has been one of the most important pathogens in commercial pig farming. Present in all major pork producing countries, it causes high economic losses as a result of the associated disease, porcine reproductive and respiratory syndrome (PRRS). PRRS is characterized by reproductive disorders in sows and respiratory disease in piglets. The severity of disease and mortality rates are highly dependent on the virulence of the virus strain involved. Of the two PRRSV species, PRRSV-1 is mainly present in Europe, whereas PRRSV-2 is predominantly distributed in North America and Asia. Their single-stranded, positive-sense RNA genomes share approximately 60% sequence identity. Both are characterized by a very high mutation rate, which is most likely partially responsible for the lack of satisfactory vaccines. The more diverse PRRSV-1 can be further classified into at least three subtypes, of which only subtype 1 occurs in Western Europe. Diagnosis of PRRSV infection is usually performed by RT-PCR or ELISA. Subsequent sequencing of the resulting small PCR products is common. Nevertheless, published information on current Austrian field strains is sparse. Also, available sequence data are not linked to the in vitro and in vivo phenotype of the viruses. Therefore, the aim of this thesis was the detailed characterization of current Austrian PRRSV field strains. To this end, virus from field samples was isolated in cell culture. Full genome sequences were obtained of two isolates (AUT13-883 and AUT14-440); another isolate (AUT15-33) was partially sequenced. Based on these sequence data, all three field isolates can be classified as PRRSV-1 subtype 1. They do not cluster with any vaccine strains currently licensed in phylogenetic analysis. For strain AUT15-33 a close relationship to Croatian strains could be determined, indicating a potential introduction from this region. It was isolated from a farm that experienced a PRRS outbreak with, for Austrian standards, severe reproductive failure in sows. During the following year, it spread to many other farms across Austria, underlining its clinical relevance. More special properties were found for strain AUT14-440, which grew unusually for a PRRSV-1 strain spontaneously on MARC-145 cells. Furthermore, it showed a distinct and unique deletion of twelve amino acids in the overlapping region of glycoprotein 3 and 4. This could potentially affect receptor binding and account for changes in the range of target cells. Also, neutralizing
epitopes might be affected by the deletion. To combine the molecular findings with the clinical outcome of infection, AUT14-440 and AUT13-883 were tested under controlled conditions in an animal trial for their degree of virulence. Both strains caused similar lung lesions but only piglets infected with AUT14-440 developed clinical disease (especially dyspnoea). Whether this result can be explained by the molecular findings remains to be elucidated. In conclusion, it was demonstrated that current Austrian PRRSV strains differ markedly from vaccines strains available on the market and an ongoing introduction of new field strains into Austria seems likely. Some field isolates show remarkable new properties that could account for an evolutionary advantage. The well-characterized new field isolates link knowledge of pheno- and genotype and will provide an excellent basis for further research on the molecular mechanisms determining PRRSV virulence.
1.2 Zusammenfassung


2. General Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is often regarded as “the most important disease in intensively raised pigs in North America and Europe” (OIE) as it causes substantial economic damage (annual losses in the US were estimated at US $560 and 664 million in 2005 and 2013 (Holtkamp et al., 2013; Neumann et al., 2005)) and also animal welfare problems. Mortality rates, although showing large variation depending on the underlying strain, can be as high as 100 % for the highly pathogenic Asian strains (Zhou and Yang, 2010).

Historically, PRRS appeared as so-called “mystery swine disease” at around the same time in Europe and North America. First reports from the United States about a disease causing both reproductive and respiratory signs in pigs date to 1987/1988. In 1990, a similar syndrome was observed in Germany and referred to as “Seuchenhafter Spätabort der Schweine” (Lindhaus and Lindhaus, 1991). On both continents the disease spread quickly to neighbouring countries, leading to the first isolation of the responsible pathogen in 1991 in Lelystad (Wensvoort et al., 1991). The typical combination of reproductive disorders and respiratory clinical signs is now reflected in the name of the disease, PRRS (Goyal, 1993).

Since its discovery about 30 years ago, the causative pathogen *Porcine reproductive and respiratory syndrome virus* (PRRSV) has spread to all major pig producing countries. During this time extensive research has been conducted for a better understanding of the principles of PRRSV infection. Nevertheless many open questions remain and the biggest pork producing countries; China, USA and Germany, still lack an efficient PRRSV control.

2.1 The virus

2.1.1 Arteriviridae

PRRSV was first isolated 1991 in Lelystad in the Netherlands (Wensvoort et al., 1991). The virus strain was therefore named Lelystad virus (LV) and served as prototype strain for the European variant of PRRSV, formerly genotype 1. Since 2015, the European variant is recognized as own species, *Porcine reproductive and respiratory syndrome virus 1*
(PRRSV-1), as is the North American variant, formerly genotype 2, which is now termed *Porcine reproductive and respiratory syndrome virus 2* (PRRSV-2). It is represented by the prototype VR-2332 (Adams et al., 2016; Nelsen et al., 1999). For the highly diverse PRRSV-1 the division into subtype 1, 2 and 3 has been suggested and widely used since (Stadejek et al., 2008). Also, PRRSV-2 can be grouped into different clades (Shi et al., 2010b). Both species belong to the genus *Arterivirus* within the family *Arteriviridae* and the order *Nidovirales* (Adams et al., 2016). In this genus, 13 species are grouped. Prominent members, apart from PRRSV, are the *Equine arteritis virus* (EAV), the *Lactate dehydrogenase-elevating virus* (LDV) and the *Simian hemorrhagic fever virus* (SHFV) (Adams et al., 2016). The remaining eight species were added only in 2015 and mainly consist of other simian viruses. The family *Arteriviridae* also contains a more distinct and therefore unassigned virus, which was found in the Australian brushtail possum and is suspected to cause neurologic disease (*Wobbly possum disease virus*) (Adams et al., 2016; Dunowska et al., 2012).

The prototype species of the family is EAV. In horses, donkeys and mules EAV may cause abortions in pregnant mares and respiratory disease in naïve animals (Holyoak et al., 2008). The infection with EAV is one of the major equine venereal diseases and is listed as a notifiable disease by the World Organization for Animal Health OIE (OIE, 2016). LDV shares a common ancestor with PRRSV and is the most closely related arterivirus. Because LDV establishes nearly asymptomatic, life-long infections in mice, it serves as a research model (Plagemann and Moennig, 1992). The virus name refers to the rise of several serum enzyme levels due to macrophage destruction in the course of infection, in general the only altered clinical parameter (MacLachlan et al., 2008).

SHFV was first isolated from macaque monkeys showing haemorrhagic fever with high fatality rates (Plagemann and Moennig, 1992). Later it was shown that also other monkeys than macaques are susceptible to SHFV but are probably inapparent carriers (Gravell et al., 1986; Palmer et al., 1968). The disease characteristics resemble those of Ebola and indeed a combined outbreak of SHFV and Ebola virus in monkeys from the Philippines imported to the US has been reported (Jahrling et al., 1990).

Arteriviruses are enveloped viruses with a diameter of 50 to 70 nm. Their genome consists of a single-stranded RNA molecule with positive polarity and is polyadenylated at the 3’-end and very likely capped at the 5’-end (Siddell and Snijder, 2008).
The most well-studied arterivirus species PRRSV-1 and -2, EAV, LDV and SHFV share several common properties. They all have a strict host tropism (MacLachlan et al., 2008) and their primary target cells are macrophages. Furthermore, they are able to cause persistent infections that “occur when the primary infection is not cleared efficiently by the adaptive immune response” and are characterized by continuous presence of virions, proteins and genomes (Flint et al., 2009). Persistent or prolonged infections of PRRSV are mostly asymptomatic (Plagemann and Moennig, 1992). However, the mechanisms of persistence, especially with regard to the immune evasion mechanisms involved, still need to be elucidated.

2.1.2 Genome structure

The genome of PRRSV-1 and -2 is about 15 kb in length and contains at least ten open reading frames (ORF) (Firth et al., 2011; Johnson et al., 2011). ORF1a and ORF1ab represent about 75 % of the genome and encode the nonstructural proteins. The resulting two polyproteins (pp) are cleaved by internal proteases into at least 14 nonstructural proteins (nsp) responsible for virus replication (Fang and Snijder, 2010). An alternative -2 ribosomal frameshift in ORF1a gives rise to the expression of another nonstructural protein, nsp2TF (Fang et al., 2012) (Fig. 1). Many nonstructural proteins interact with the host’s innate immunity. Other key functions are the protease activity of nsp1a, nsp1b, nsp2 and the main serine protease nsp4, the RNA-dependent RNA-polymerase in nsp9, the helicase function of nsp10 and the nidovirus-specific endoribonuclease in nsp11. Nsp2 represents the longest and most variable protein of PRSSV, prone to deletions and insertions (Yun and Lee, 2013).

![Fig. 1. Genome organisation of PRRSV. On top: open reading frames and genome modifications. Below: translation pattern with internal cleavage sites marked by arrows (in blue: nonstructural proteins, in orange: structural proteins)
ORF2-6 code for the structural proteins that form the virus envelope. The structural proteins with the highest and lowest sequence diversity, glycoprotein 5 (GP5) and membrane protein (MP), are encoded by ORF5 and ORF6 and act as a disulfide-linked heterodimer (Murtaugh et al., 2010; Yun and Lee, 2013). These two major membrane proteins are crucial for the production of viral particles (Wissink et al., 2005). Furthermore, the recently discovered ORF5a encoded protein was shown to be essential for virus viability (Johnson et al., 2011; Sun et al., 2013). ORF2, ORF3 and ORF4 code for the minor membrane proteins GP2, GP3 and GP4. They form a heterotrimer abundantly present on the virus surface, which is very likely involved in virus entry into cells as it is responsible for cell tropism and binds to the proposed virus receptor CD163 (Loving et al., 2015; Tian et al., 2012). For EAV it was also shown that interactions between the membrane proteins GP2, GP3, GP4, GP5 and MP play a major role in determination of cell tropism (Go et al., 2010). The small envelope protein (SEP), encoded by ORF2b, is also associated with GP2, 3 and 4 through non-covalent interactions. Like all other minor membrane proteins, it is essential for infectivity of virions (Wissink et al., 2005) but otherwise its function remains unclear. Finally, the most abundantly expressed structural protein, the nucleocapsid protein (NP), is encoded by ORF7. It occurs as a dimer that binds the viral RNA with its N-terminal half and forms the capsid with the C-terminal half (Dokland, 2010).

2.1.3 Attachment and entry

Similar to its host restriction, PRRSV also shows a very narrow cell tropism. In vivo mainly macrophages are targeted, especially porcine alveolar macrophages (PAM) (Yun and Lee, 2013). In cell culture only one continuous cell line supports growth of certain PRRSV isolates: the green monkey kidney cell line MA-104 and its derivatives, e.g. MARC-145 (Kim et al., 1993). These growth characteristics give an indication on the receptors involved in virus entry: MA-104/MARC-145 cells express CD163, which is otherwise typical for macrophages. Furthermore, CD163-expression in formerly non-permissive cell lines results in susceptibility to PRRSV (Calvert et al., 2007). Both of these findings do not apply for CD169 (sialoadhesin), which was also reported to play a critical role but was recently shown not to be required for virus binding and internalization by analysis of knockout pigs (Prather et al., 2013). In contrast, it could be shown that CD163-knockout pigs were resistant to PRRSV (Whitworth et al., 2016), underlining the key role of this receptor that interacts with the minor membrane proteins GP2 and GP4 (Das et al., 2010). Probably also other factors such as
heparan sulfate and cellular proteases are supportive for attachment and internalization of the virus (Van Breedam et al., 2010). After clathrin-mediated endocytosis, the viral genome is released into the cytoplasm where the replication takes place.

### 2.1.4 Replication

Like all other nidoviruses, PRRSV generates a nested set of subgenomic mRNAs (Meulenberg et al., 1994). The process of replication begins with ORF1a and ORF1b being translated by host ribosomes into pp1a and pp1ab. After autocatalytic cleavage the nonstructural proteins assemble into membrane-associated replication and transcription complexes (RTC) that amplify the viral genome (= replication) and synthesize the subgenomic mRNAs (= transcription). At first complete and subgenomic RNA molecules with negative polarity are produced, then they are transcribed into a positive-sensed viral genome and subgenomic mRNAs. The characteristic subgenomic mRNAs are subsequently translated into the structural proteins. The nucleocapsid is assembled from NP molecules that bind the positive-sensed viral genome and then buds at intracellular membranes. Hereby it acquires its envelope consisting of the other structural proteins. The progeny virions are released through exocytosis (Faaberg, 2008; Yun and Lee, 2013).

Even for RNA viruses, PRRSV shows an extensive genetic variability, with PRRSV-1 exceeding that of PRRSV-2. The evolutionary rate was estimated to 4.7-9.8 x 10^{-2} sites/year, which was the highest rate reported for an RNA virus so far. The reasons are not fully understood. One possible explanation is the infidelity of the RNA polymerase, which has no proofreading mechanism. As a consequence, PRRSV has a high mutation rate and occurs in its host as a virus cloud rather than as a single strain. As most RNA polymerases of RNA viruses lack proofreading abilities, other mechanisms have to be at work to create this outstanding variability. It was suggested that PRRSV possesses a higher replication frequency than other viruses. Another contributing factor might be recombination, which is inherent in PRRSV replication cycle when subgenomic mRNA is formed (Hanada et al., 2005; Murtaugh et al., 2010). Either way, the “ever-expanding diversity” (Murtaugh et al., 2010) of PRRSV complicates diagnostics, vaccine development and epidemiological studies.
2.2 The disease

2.2.1 Clinical manifestations

The clinical picture of PRRS is highly variable and strongly depends on the virus strain involved as well as the age and immune status of the host.

Adults are mostly inapparently infected or show mild clinical signs such as lethargy, fever or anorexia. Therefore, a “rolling inappetence” accompanies the spread in a herd. Sometimes, a typical cyanotic discolouring of ears or vulvae (“blue ear disease”) can be seen (Goyal, 1993; Nodelijk, 2002). Reproductive failure of sows highly depends on the gestation stage. Infection in early or mid-gestation is often unproblematic, whereas sows infected in late gestation abort or give birth to mummified, autolytic, stillborn or weak piglets (Kranker et al., 1998; MacLachlan et al., 2008).

Nursery or finishing pigs show reduced growth rates and elevated mortality as a consequence of mild to severe respiratory disease (Cho and Dee, 2006). Mouth-breathing, nasal discharge, conjunctivitis, sneezing, fever and listlessness may occur. The younger the animals, the more severe the clinical signs are. Pre-weaning mortality can therefore be as high as 50-60%. Additionally, PRRS is often complicated by secondary infections (Goyal, 1993; Nodelijk, 2002).

2.2.2 Co-infections

Major complications in the field occur as a result of secondary infection. In theory, all swine pathogens can establish a co-infection with PRRSV. Specific interactions that aggravate respiratory disease have been described between PRRSV and PCV2 (Harms et al., 2001; Rovira et al., 2002), PRRSV and *Mycoplasma hyopneumoniae* (Thacker et al., 1999) and PRRSV and *Bordetella bronchiseptica* (Brockmeier et al., 2001). Interestingly, a recent retrospective study from Austria reported that weaning piglets with a fungal *Pneumocystis* lung infection are less likely to be PRRSV co-infected (Weissenbacher-Lang et al., 2016).

2.2.3 Highly pathogenic strains
PRRSV-1 infections appear to be in general less severe with regard to clinical signs compared to PRRSV-2. This is accompanied by lower levels of viremia and complicates the development of reproducible animal models (Murtaugh and Genzow, 2011). However, PRRSV-1 subtypes 2 and 3 originating from Eastern Europe are reported to cause more severe clinical disease (Karniychuk et al., 2010; Morgan et al., 2013). Subtype 3 strain Lena was described as “highly pathogenic” and differed from subtype 1 strains not only in the degree of clinical disease and in the level of viremia (up to $10^6 \text{TCID}_{50}/\text{ml}$) but also in antigenic properties (Karniychuk et al., 2010). In contrast, the subtype 3 strain SU1-bel did not cause higher levels of viremia. Nevertheless, clinical signs were severe, which could be explained by an enhanced inflammatory immune response due to higher IFN-$\gamma$ levels (Morgan et al., 2013).

In 2006, a new form of highly pathogenic PRRSV-2 strains emerged in China. In contrast to the PRRS characteristics known so far, not only young pigs developed severe disease but also sows. Affected pigs showed high fever (40-42°C), neurological signs and rash. Altogether 400,000 pigs died in the epidemic within a short time period. Molecular analysis revealed that all PRRSV strains involved in this outbreak displayed a typical deletion of 30 amino acids (aa) in nsp2 (Tian et al., 2007). However, it could be shown that the deletion was not responsible for the virulence of these highly pathogenic PRRSV strains (Zhou et al., 2009). So far, despite extensive research, no definite virulence factors could be identified for PRRSV (An et al., 2011). However, it is likely that the minor envelope proteins GP2-4, which seem to determine cell tropism (Tian et al., 2012), may influence virulence of virus strains.

### 2.2.4 Persistence

A hallmark of arteriviruses is their ability to establish persistent, in general clinically inapparent infections (MacLachlan et al., 2008). For PRRSV a “smoldering” type of infection was described, marked by a continuous virus replication at low levels for up to several months (Allende et al., 2000; Wills et al., 2003). With regard to the mechanism of virus persistence there is evidence that the virus’ immune evasion capabilities prevent an effective immune response of the host. Possible mechanisms include glycosylation of GP5 (Wei et al., 2012b) and a so-called ‘decoy epitope’ that may be retained in some GP5 molecules after signal peptide cleavage (Thaa et al., 2013). Studies on involvement of GP3 in glycan shielding yielded inconsistent results (Vu et al., 2011; Wei et al., 2012a).
2.3 Pathogenesis

2.3.1 Virus replication in the host

After intranasal, oral, venereal or intrauterine infection, the virus initially replicates in local permissive cells such as alveolar or other tissue macrophages. The viremic phase starts very soon after infection (pi) and may last up to one month. Levels of viremia are in general lower for PRRSV-1 field strains (Murtaugh and Genzow, 2011). After this acute stage the infection becomes persistent. The virus replicates at low levels, mainly in lymphoid tissues, and may be shedded and transmitted for at least two months pi. Until the virus is eventually cleared from the host it can take up to eight months (Bierk et al., 2001; Lopez and Osorio, 2004; Rowland et al., 2003; Wills et al., 2003).

The process of transplacental infection of foetuses and subsequent foetal death or abortions is not fully understood yet. PRRSV efficiently crosses the placenta only in late gestation. Potential reasons, as for example a lack of susceptible cells in the placenta or factors that prevent PRRSV replication in the endometrium, still have to be proven (Karniychuk and Nauwynck, 2013). Likewise, the reproductive failure observed when PRRSV infection takes place in late gestation cannot be fully explained yet, although it seems to be rather an effect of placental tissue damage than a direct effect of virus growth in the foetus. A possible explanation is that virus replication in the foetal part of the placenta causes mesenchymal damage at the maternal-foetal interface. Apoptosis and necrosis of infected cells leads to degeneration of the fetal placenta that is not compatible with normal foetal development (Karniychuk et al., 2011; Karniychuk et al., 2012).

2.3.2 Histopathology of PRRSV infection

Histopathological changes in the uterus of the infected sow include lymphoplasmacytic and histiocytic endometritis with perivascular cuffing, vasculitis and oedema in myo- and endometrium (Novakovic et al., 2016). In contrast, histopathological lesions in foetuses are rare. In the lung of pigs affected by the respiratory form of PRRS, hypertrophy and hyperplasia of pneumocytes type II, septal infiltration by mononuclear cells, perivascular and intraalveolar accumulation of inflammatory cells as well as alveolar necrotic debris are common findings. Accompanying gross lesions are enlarged lymph nodes, consolidation of lung tissue and periocular oedema (Balka et al., 2013; Rossow, 1998).
2.4 Diagnostics

2.4.1 Direct detection of PRRSV

As a consequence of viremia and replication in the lung during the acute phase of PRRSV infection, serum and lung lavage or tissue samples are ideal for diagnosis of PRRSV infection. The most common method is reverse-transcription polymerase chain reaction (RT-PCR). Apart from confirmation of clinical disease, it is the recommended method to declare a population or an individual free from infection and is therefore widely used for surveillance (OIE, 2012). Other methods for direct detection of the pathogen are virus isolation, immunohistochemistry or in situ hybridisation.

Virus isolation is likewise recommended for confirmation of clinical cases but rarely used because the isolation process can be very difficult. Not all PRRSV strains infect the continuous cell lines derived from MA-104 green monkey kidney cells. Especially PRRSV-1 strains usually grow very poorly or not at all on these cells without adaptation (MacLachlan et al., 2008; OIE, 2012). Therefore the use of primary cells, porcine alveolar macrophages (PAM), which are isolated from pig lungs, is recommended. PAM should only be collected from young pigs with a high health status (especially tested to be PRRSV-negative) and should be evaluated for their susceptibility before use (OIE, 2012). PRRSV grows with a cytopathic effect (Mengeling et al., 1995; Wensvoort, 1993).

Immunohistochemistry and in situ hybridisation are rarely used in diagnostic routine but are suitable for confirmation of clinical disease on formalin-fixed tissue. In situ hybridisation is complicated by the vast genetic diversity of PRRSV caused by high mutation rates and recombination (OIE, 2012).

2.4.2 Indirect detection of PRRSV infection

For indirect detection of PRRSV infection a variety of serological tests have been described to date. Possible samples are serum, oral fluid and meat juice.

The most common assay is an ELISA that detects early, non-neutralizing antibodies directed against N protein of PRRSV-1, PRRSV-2 or both. Several commercial kits are available that
show good sensitivity and specificity and are widely used for surveillance purposes. Other options are the immunoperoxidase monolayer assay and the immunofluorescence assay with PAM or MARC-145 cells (OIE, 2012).

2.4.3 Sequencing and phylogenetic analysis

Sequencing of RT-PCR products is commonly used to differentiate field from vaccine strains, to trace the source of virus introduction, to identify virus strains known to be especially virulent or to follow PRRSV evolution. The latter is crucial for development or evaluation of vaccines and diagnostic methods. For example, mutations in ORF7 of different PRRSV-1 subtypes can hamper primer binding and therefore affect the reliability of RT-PCR diagnostics (Stadejek et al., 2008). This is especially important for PRRSV-1 subtype 2 and 3, as most PCR protocols were originally developed for subtype 1 strains. Therefore conventional kits and in-house assays may fail to detect subtype 2 and 3 (Wernike et al., 2012).

Sequencing is mainly done for short sequences, namely ORF5 (603-606 bp) or ORF7 (372-387 bp). ORF5 was chosen because of its variability and its assumed impact on viral pathogenesis and immunity (Murtaugh et al., 2010). Based on the ORF7 length, a subtyping system for PRRSV-1 was introduced (Stadejek et al., 2008). This was called for by the high diversity of PRRSV-1 isolates. The three different subtypes – pan-European subtype 1 (ORF7 = 128 aa) and Eastern European subtype 2 (ORF7 = 125 aa) and 3 (ORF7 = 124 aa) – differ not only genetically but also in their virulence (Karniychuk et al., 2010; Morgan et al., 2013). Subtyping based on ORF5 or ORF7 normally leads to homologous results, except for some strains from Eastern Europe (Shi et al., 2010a; Stadejek et al., 2013). A possible explanation would be recombination events, which are known to occur in this genomic region and should be assessed by breakpoint analysis (Shi et al., 2010a; van Vugt et al., 2001). For PRRSV-2, though much less diverse, multiple genetic lineages have been defined by extensive phylogenetic analysis (Shi et al., 2010b).

With phylogenetic analysis of the obtained sequences, the relationship between different strains can be predicted. Unfortunately, results of phylogenetic analysis do not correlate with immunological relatedness and thus cannot predict cross-protection between strains (Murtaugh and Genzow, 2011).
2.5 Epidemiology and control

2.5.1 Origin of PRRSV

The first isolation attempts in Europe and in North America soon revealed that the different strains from both continents showed a similar disease pattern but had a very low sequence identity (around 60%) with each other. Very recently, they were classified as two different species; PRRSV-1 and PRRSV-2 (Adams et al., 2016). Their marked divergence suggests that they were geographically separated a long time before their appearance. Why these two species then became clinically apparent nearly simultaneously on two different continents remains a matter of speculation. One explanation is a major change in swine husbandry around that time, which lead to indoor swine keeping with high animal densities, increased transportation as a consequence of multi-site production and the introduction of artificial insemination (MacLachlan et al., 2008; Murtaugh et al., 2010). Thus, conditions for transmission were improved, allowing for expansion of its geographic reach.

2.5.2 Transmission of PRRSV

With regard to transmission routes of PRRSV, the importance of the changes in swine husbandry becomes apparent. The virus is mainly transmitted horizontally by direct contact through body fluids and excretions such as saliva, nasal secretions, semen or milk (MacLachlan et al., 2008). Hence high pig densities, mixing of pigs from different sites and a high degree of artificial insemination may well have contributed to the rise of PRRS. Another central route of transmission is the vertical, transplacental transmission. It is responsible for the most devastating clinical signs of PRRS: abortions and stillborn or weak piglets.

2.5.3 Control strategies

PRRSV can also be spread indirectly by transport vehicles, insects, needles or aerosols (Cho and Dee, 2006). As a consequence, strict biosecurity protocols have to be applied to protect a PRRSV-naïve herd. Furthermore, purchase of pigs should be restricted to herds with a tested PRRSV-free status. Additionally, animals should be kept in quarantine before introduction into the herd and semen from boars used for breeding has to be analysed regularly. Nevertheless, PRRSV (re-)introduction into formerly free herds is common, raising the controversially discussed question concerning the impact of airborne transmission (Alonso et al., 2015; Otake et al., 2010).
Eliminating PRRSV from an infected herd is very challenging. Protocols that describe total depopulation, test and removal or herd closure are available (Dee and Molitor, 1998; Dee et al., 1997; Torremorell et al., 2003). Herd closure has been shown to eliminate PRRSV from large herds when all animals are exposed to virus and the herd is afterwards closed for at least 200 days (Corzo et al., 2010). However, the measures described are very costly. Vaccination is therefore very common in middle-sized pig farms with pig-producing, nursery or fattening units.

2.6 Vaccines

2.6.1 Inactivated and live attenuated vaccines

A variety of inactivated as well as live attenuated (modified live vaccines, MLV) vaccines are available. By adaptation and passage on a continuous cell line (e.g. MARC-145), PRRSV-1 isolates loose part of their virulence for PAM; a process often used for MLV development (Renukaradhya et al., 2015). Inactivated vaccines lack efficacy in inducing a protective immune response and are therefore, in spite of their safety, not very common (Zuckermann et al., 2007). In contrast, MLV are able to induce long-lasting protection but do not always show full protection against heterologous strains, which is, with respect to the high genetic variation within one PRRSV species, a major problem (Kimman et al., 2009). Especially for PRRSV-1 there are concerns in terms of protection against distinct, heterologous strains (Labarque et al., 2004). Unfortunately, genetic similarity could not be linked to cross-immunity (Prieto et al., 2008). Heterologous cross-protection is existing but variable and is unfortunately not predictable yet (Murtaugh and Genzow, 2011). Additional concerns with regard to MLV are reversion to virulence and recombination with circulating virus (Amonsin et al., 2009; Botner et al., 1997; Li et al., 2009).

Efficacy of MLV has been proven for PRRSV-1 and -2 (Renukaradhya et al., 2015), even though vaccinated herds may experience an outbreak (Rossow et al., 1999). MLV do not prevent PRRSV infection but they may reduce virus shedding and transmission of field virus strains (Rose et al., 2015). Other beneficial effects described are reduction of lung lesions and clinical signs (Dwivedi et al., 2011). As MLV strains replicate in their hosts, they may be shed and transmitted vertically and horizontally. Because of the potential to revert to
virulence, safety of MLV has to be evaluated carefully before approval (Renukaradhya et al., 2015; Scortti et al., 2006).

2.6.2 Vaccination schemes

To prevent transplacental infection and subsequent death of foetuses, sows are usually vaccinated at a regular interval. Common schemes recommend vaccination every 3-5 months or at day 60 of gestation and at day 6 after birth (6/60 scheme) (Grosse Beilage et al., 2009; Papatsiros et al., 2015). This means that sows are vaccinated up to four times a year; a very costly and time-consuming procedure, which is also disturbing for the sows.

A controversially discussed question is whether a herd experiencing a PRRSV outbreak should be vaccinated with MLV. Vaccination is often used as a therapeutic intervention due to the lack of other pharmaceutical possibilities. A reduction of field virus shedding after vaccination was proven (Cano et al., 2007; Linhares et al., 2012) but some authors regard this effect as negligible (Amadori and Razzuoli, 2014). Because PRRSV field and vaccine strains are present in the herd at the same time, concerns regarding the possibility of recombination have been raised (Li et al., 2009). Also, escape mutants can arise as a result of evolutionary selection pressure (Costers et al., 2010).

2.6.3 New vaccination concepts

Apart from MLV and inactivated vaccines, other vaccination strategies have been discussed; for example subunit vaccines, vector vaccines or generation of DNA vaccines using reverse genetics. There is also a need for a combined vaccine–serological testing–system that allows the differentiation between vaccinated and naturally infected animals. All these possibilities have been in the focus of extensive research but are not or underrepresented on the market to date (Murtaugh and Genzow, 2011; Renukaradhya et al., 2015).

So far, there is no vaccine available that is both safe and effective against various PRRSV strains. Due to the high evolutionary rate of PRRSV, the development of novel immunization strategies based on improved knowledge of the mechanisms underlying protective immunity is pivotal.
2.7 Immunity

One reason for the lack in efficacious vaccines is the variable and often weak immune response to PRRSV infection in general, naturally or vaccine-induced. Nevertheless, the host normally clears the virus after a prolonged infection that can last several months. In addition, the success of the herd closure procedure demonstrates that a sterilizing and complete immunity against PRRSV can develop (Loving et al., 2015).

2.7.1 Humoral immunity

The role of the humoral immune response is not clear but most likely not of major importance. Under experimental conditions, viremia was resolved without detectable levels of neutralizing antibodies (nAb) in the blood and pigs without nAb were resistant to re-infection (Diaz et al., 2006). These findings indicate that other mechanisms apart from humoral immunity must play a crucial role in protection against PRRSV. However, passive transfer of neutralizing antibodies protects sows and, when applying a higher dosage, also piglets against infection (Lopez et al., 2007).

Whereas the first antibodies, directed against the most abundant N protein, are already detected one week post infection (pi), nAb appear not earlier than four weeks pi – too late to prevent a chronic infection. However, nAb present in a sufficient amount before the infection occurs may prevent subsequent infection (Loving et al., 2015). Maternal antibodies can be detected up to 4-8 weeks after birth (OIE, 2012).

The major neutralizing epitopes are most likely located on the structural proteins known to interact with the host cell: GP3 (Vanhee et al., 2011), GP4 (Vanhee et al., 2010), GP5 (Weiland et al., 1999) and MP (Yang et al., 2000). The role of nAb directed against GP5 is controversially discussed because the suggested essential role of GP5 in cell entry could not be proven and therefore it probably does not contain key sites for blocking of cell entry (Murtaugh and Genzow, 2011). In analysis of PRRSV-1-antisera, no virus-neutralizing antibody targets were found in GP5, instead the largest number of antigenic regions was found in GP3 (Vanhee et al., 2011). Additional evidence for the minor role of GP5 in immune protection comes from subunit immunizations with this protein that did not result in protection but in enhanced clinical signs upon challenge (Prieto et al., 2011). In contrast, the minor envelope proteins, including GP4, interact with the proposed PRRSV receptor CD163
(Das et al., 2010). Consequently nAb targeting GP4 could prevent infection of cells (Loving et al., 2015). Furthermore, the development of GP4-escape mutants has been described, indicating that the region is under selection pressure and plays a part in PRRSV evolution (Costers et al., 2010).

2.7.2 Cell-mediated immunity

One reason for the inability of pigs to clear PRRSV infections rapidly might be an insufficient T-cell response. Unfortunately, information on cell-mediated immunity directed against PRRSV infection is limited and inconclusive.

The onset of cell-mediated immunity seems to be delayed and the impact on virus clearance could not be proven (Butler et al., 2014; Loving et al., 2015; Meier et al., 2003). Although there is evidence for a PRRSV-related CD8+ T-cell response and CD8+ T-cells are increased in infected lungs and lymphoid tissue (Ferrari et al., 2013; Gomez-Laguna et al., 2009; Samsom et al., 2000), the link to control of infection remains to be established. CD3+CD8high PBMCs of PRRSV-infected pigs did not show cytotoxic activity towards PRRSV-infected alveolar macrophages (Costers et al., 2009).

Frequency of T-cells in general is not increased by PRRSV infection (Klinge et al., 2009). Observations on whether the frequency of regulatory T-cells is increased or not are inconsistent (Rodriguez-Gomez et al., 2015; Silva-Campa et al., 2012; Silva-Campa et al., 2010).

Efforts have been made to determine potential T-cell targets. For PRRSV-1 and PRRSV-2, immunodominant epitopes were identified in a broad range of proteins. The most important epitopes for PRRSV-1 seem to be within the structural proteins GP5, MP and NP and within the non-structural proteins nsp1β, nsp2 and nsp5 (Burgara-Estrella et al., 2013; Diaz et al., 2009; Mokhtar et al., 2014).

2.7.3 Innate immunity

Many studies have been conducted to elucidate the impact of the innate immune response on PRRSV infection. The results are controversial and vary greatly for different virus strains. In most cases, PRRSV seems to suppress the production of the type 1 interferons (IFN) IFN-α.
and IFN-β (Lee et al., 2004; Luo et al., 2008; Sun et al., 2012). By contrast in other studies a strong innate immune response was detected (Liu et al., 2010). Viral proteins responsible for the interaction with factors of the innate immune response are nsp1α, nsp1β, nsp2 and nsp11 (Han and Yoo, 2014). Evidence exists that the virus is sensitive to IFN-α, IFN-β and IFN-γ (Garcia-Nicolas et al., 2014; Lee et al., 2004).

Despite all open questions regarding a protective immune response against PRRSV and immune modulation mechanisms of the virus, it is evident that PRRSV is very successful at evading the host’s immune response for a prolonged time. This persistence hampers control of the pathogen, especially in pig-dense areas where virus re-introduction is very common.

2.8 PRRSV in Austria

Austria’s geographic situation promotes the occurrence of pathogens from all over Europe. Situated between Western and Eastern Europe, it shares borders with eight countries and contains major transit routes. Western Europe is free of many animal diseases that trouble parts of Eastern Europe, such as African Swine Fever, Rabies, Equine Infectious Anaemia and also PRRSV-1 subtype 2 and 3 or PRRSV-2. Hence surveillance in Austria is critical not only for the country itself but also for the rest of Europe.

2.8.1 Prevalence of PRRSV in Austria

Epidemiological data on the prevalence of PRRSV in Austria is rather limited. In 1994, a case report documented one of the first Austrian PRRS outbreaks, which was mainly characterized by reproductive problems such as late-term abortions (Krassnig et al., 1994). Investigations on pathogens involved in respiratory disease in pigs from 2004 to 2007 identified PRRSV as the most important pathogen (Elicker et al., 2009). Data from a serological survey from 2006 to 2009 suggests that PRRSV is present in about three-quarters of all farms (Nagl, 2010). This is comparable to other European countries, where the seroprevalence seems to exceed 50% in most cases (Duinhof et al., 2011; Evans et al., 2008; Lopez-Soria et al., 2010; Meemken et al., 2014; Powell et al., 2016). However, one has to keep in mind that, intended or not, most studies include data from vaccinated pigs, too.
2.8.2 PRRSV sequence data from Austria

Slightly more information is available on PRRSV strains circulating in the field in Austria. Published sequences include one ORF6-7 sequence from 2002 and 21 ORF5 sequences from 2005 (Indik et al., 2005; Revilla-Fernandez et al., 2005), seven strains with incomplete ORF5 and complete ORF7 sequences from 2008 (Jackova et al., 2012; Jackova et al., 2013), three ORF7 sequences from 2007 (Balka et al., 2010) and one strain with ORF5 and ORF7 sequence from 2015 (Korschineck et al., 2015). Available sequences on GenBank are summarized in Table 1.

### Table 1 Published PRRSV sequences from Austria

<table>
<thead>
<tr>
<th>Publication</th>
<th>Sequences with accession number, length and name of strain</th>
<th>Collection/ Submission date</th>
</tr>
</thead>
</table>
| Korschineck et al., 2015. Porcine reproductive respiratory syndrome virus (PRRSV) strains detected in domestic pigs in Austria. Unpublished.                                                                 | KT265738 633 bp "Acro"  
KT265737 459 bp "Acro"                                                                                                                              | 2015                         |
| Balka et al., 2010. PriProET based melting point analyses on PRRSV positive field samples. Mol Cell Probes 24, 411-414.                                                                                         | GU930371 - GU930373 387 bp "AUT1 – AUT3"                                                                 | 2009                         |
KF134447 – KF134453 387 bp "LA1 – LA10"                                                                                                           | 2008                         |

Except for four ORF5 sequences from 2005 with high identities to PRRSV-2 vaccine strains (Indik et al., 2005), only PRRSV-1 subtype 1 has been detected in Austria. Comparably, in the rest of Europe PRRSV-2 was mostly associated with the use of PRRSV-2 MLV strains
(Balka et al., 2008; Botner et al., 1997; Stadejek et al., 2013). In Austria, a PRRSV-2 MLV has never been licensed. Therefore the PRRSV-2 sequences are likely to result from imported pigs.

All data presented in Table 1 were obtained by RT-PCR only and no virus isolation has been done. As a consequence an evaluation of the biological properties of the strains is not possible. Another disadvantage is that only short ORF5 or ORF7 sequences were determined, in the majority of the cases not even both, which complicates phylogenetic analysis enormously. Additionally, the big majority of sequences was obtained eight or ten years ago and so topicality is lacking.

Complete genome sequences are not only missing in Austria but are sparse for PRRSV-1 in general. Of 303 full PRRSV genome sequences in GenBank only 14 represent PRRSV-1 strains (Lu et al., 2014). This is due to the fact that most research on PRRSV is done in the US and China; countries that are mainly affected by PRRSV-2.
2.9 Aims and Hypotheses

PRRSV is a pathogen of major importance due to its impact on economy and animal welfare. The control relies heavily on vaccination. Available vaccines are based on strains isolated decades ago and have failed to control the disease in many cases. Since PRRSV is a RNA virus with a high mutation rate, it is likely that current field strains differ significantly from employed vaccine strains. Therefore, vaccine efficacy might be reduced. Furthermore, novel properties influencing the virulence of virus strains could arise as a consequence of high evolutionary speed and selection pressure. However, it is difficult to substantiate the antigenic drift because there is few published data on current PRRSV strains in Austria as well as in neighbouring countries. Most existing data only include genomic information and no other virus or disease characteristics. Therefore, it was our aim to detect PRRSV strains currently circulating in the field and to characterize them. We hypothesized the following:

1) PRRSV strains currently circulating in Austria differ substantially from Lelystad-virus-like and vaccine strains, which were isolated between 10 and 25 years ago. In phylogenetic analysis they cluster in different lineages.

2) There is a repeated, independent introduction of novel PRRSV strains into Austria instead of an ongoing evolution based on few parental strains. The novel strains represent new lineages compared to the strains already described.

3) The novel PRRSV strains possess new beneficial properties giving them an evolutionary advantage. Potential novel properties will be assessed in terms of genomic sequence, phylogenetic background, in vitro growth characteristics and clinical manifestation.
3. Manuscripts

3.1 Characterization of two Austrian porcine reproductive and respiratory syndrome virus (PRRSV) field isolates reveals relationship to East Asian strains
Characterization of two Austrian porcine reproductive and respiratory syndrome virus (PRRSV) field isolates reveals relationship to East Asian strains

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Abstract
Porcine reproductive and respiratory syndrome virus (PRRSV) causes major problems for the swine industry worldwide. Due to Austria’s central location in Europe, a large number of animals are transported through the country. However, little is known about current PRRSV strains and epidemiology. We determined full-length genome sequences of two Austrian field isolates (AUT13-883 and AUT14-440) from recent PRRSV outbreaks and of a related German isolate (GER09-613). Phylogenetic analysis revealed that the strains belong to European genotype 1 subtype 1 and form a cluster together with a South Korean strain. Remarkably, AUT14-440 infected the simian cell line MARC-145 without prior adaptation. In addition, this isolate showed exceptional deletions in nonstructural protein 2, in the overlapping region of glycoprotein 3 and 4 and in the 3′ untranslated region. Both Austrian isolates caused similar lung lesions but only pigs infected with AUT14-440 developed clear clinical signs of infection. Taken together, the genetic and biological characterization of two novel Austrian PRRSV field isolates revealed similarities to East Asian strains. This stresses the necessity for a more detailed analysis of current PRRSV strains in Europe beyond the determination of short ORF5 and ORF7 sequences.

Introduction
Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically important pathogens in the swine industry worldwide [1, 2]. It is the etiological agent of porcine reproductive and respiratory syndrome (PRRS), which is characterized by respiratory disorders as well as by growth retardation in growing pigs and reproductive failure in late gestation sows [3, 4]. The disease emerged in the late eighties in North America, with the first outbreaks in Europe recorded in 1990 [3].

The causative agent, PRRSV, was first isolated in 1991 in the Netherlands [4]. This strain, Lelystad virus (LV), is regarded as the prototype strain of European PRRSV type 1 (PRRSV-1), whereas VR2332 represents the North American PRRSV type 2 (PRRSV-2). The two genotypes share only about 60% identity at the nucleotide level [5]. Due to high mutation and recombination rates [6], variability is also high within the genotypes, especially in type 1. Therefore, three subtypes have been proposed for PRRSV-1 based the size of the nucleocapsid protein (N): pan-European subtype 1 and Eastern European subtypes 2 and 3 [7].

PRRSV is a small, enveloped virus with a single-stranded positive-sense RNA genome that belongs to the family Arteriviridae, order Nidovirales [8]. The 5′-capped and 3′-polyadenylated genome of PRRSV is about 15 kb in length and contains ten open reading frames (ORF) [9, 10]. ORF1a and 1b constitute over 75% of the viral genome and encode two polyproteins, which are cleaved into at least 14 nonstructural proteins (nsp) that are responsible for genome replication and transcription...
and antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). Two additional antibiotics (enrofloxacin and kanamycin) were added for the culture of PAM. All cells were maintained at 37 °C and 5% CO₂ and observed daily for cytopathic effects.

**Sample collection**

Serum samples were collected from two medium-sized piglet producers in Upper Austria in November 2013 (farm 883) and March 2014 (farm 440). Both farms were experiencing PRRSV-related problems such as pneumonia, wasting, variance in growth, conjunctivitis and coughing in rearing piglets, as well as reproductive disorders such as mummification and abortions in sows. Whereas one farm (883) had experienced minor problems related to PRRSV over a period of 2 years, the other (440) was formerly free of PRRSV and had losses of up to 50% in one farrowing badge. Serum samples from both farms had been tested positive for PRRSV by qRT-PCR by the Animal Health Service Upper Austria. Neither of the farms had used vaccines against PRRSV.

**Virus isolation and titration**

For virus isolation, 50 µL serum of diseased piglets from farms 440 and 883 were used to inoculate 5 × 10⁶ PAM on a six-well format. After 2 days, the cell culture supernatant was clarified by centrifugation (5 min, 3000 g) and passaged on PAM and MARC-145 cells. Virus infection was generally analysed 2 days post-infection by immunofluorescence staining. Virus titres of supernatants were confirmed on PAM from a single animal to ensure comparability and expressed as tissue culture infectious dose (TCID₅₀). In the animal trial a viral stock from the 6th to 8th passage was used for inoculation.

**Detection of PRRSV-positive cells**

Cells were fixed with 1:2 methanol-acetone for 2 min at room temperature and air-dried. Anti-PRRSV-N-protein monoclonal antibodies (clone P10/b1) [20] (kindly provided by A. Saalmüller, Vienna) were employed for the detection of infected cells. Goat anti-mouse conjugated with Cy3 (Dianova/Jackson, Hamburg, Germany) was used as a secondary antibody.

**Determination and analysis of full-length genome sequences**

Total RNA was extracted from PAM 5 days after inoculation and from serum samples using the RNeasy Mini Kit and the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 30 µL distilled water and either used immediately for RT-PCR or stored at −80 °C for subsequent analysis.
RT-PCR was carried out using the LongAmpKit (NEB, Ipswich, USA) or the One Step RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Seven primer pairs that were highly conserved in PRRSV-1 strains were designed (available upon request) based on published sequences available in NCBI GenBank. The resulting PCR amplicons were overlapping and covered the whole genome.

The DNA fragments were subjected to gel electrophoresis, purified by the pepGOLD Gel Extraction Kit (Peqlab, Erlangen, Germany) and sequenced by commercial laboratories (Microsynth Austria, Vienna, Austria and Eurofins Genomics, Ebersberg, Germany). Primer selection for sequencing was based on available PRRSV-1 sequences. The determined full-length genome sequences of GER09-613, AUT14-440 and AUT13-883 were submitted to GenBank (KT344816, KT334375 and KT326148).

Initial phylogenetic analysis was carried out with NCBI’s Basic Local Alignment Search Tool for nucleotides (BLASTn). The two closest neighbours of the isolates presented in this study were determined for ORF5 and ORF7. Pairwise comparison and identity calculations were carried out with CLC Main Workbench 7.6 (CLCBIO, Aarhus, Denmark). Alignments and phylogenetic trees were generated with the software CLC Sequence Viewer 7.6 (CLCBIO, Aarhus, Denmark) with bootstrap values based on 1000 replicates. All PRRSV-1 strains with full genome sequences deposited in GenBank were used to construct the phylogenetic trees. For the trees based on ORF5 and ORF7, the two closest neighbours—as determined by NCBI BLASTn—were added. The PRRSV-2 prototype VR-2332 was used as an out-group. Recombination analysis was performed with the recombinase analysis tool (RAT) [21] using the full genome alignment of PRRSV-1 strains.

**Animal trial**

**Animals**

Twenty-seven-week-old conventional pigs (#1–20) were obtained from a PRRSV-negative herd and housed in a biosafety level 2 facility. They had been vaccinated against PCV-2 and *M. hyopneumoniae* in their third week of life. To confirm their PRRSV status nasal swabs taken at the time of arrival were analysed by qRT-PCR and serum samples were tested for antibodies against PRRSV with the commercial IDEXX X3 ELISA (IDEXX laboratories, Westbrook, USA). The animal experiments were approved by the ethics committee of the University of Veterinary Medicine, Vienna and the Federal Ministry of Science, Research and Economy (BMWF-68.205/0196-WF/V/3b/2014).

**Serum titration**

Tenfold dilution series of sera were prepared and transferred to naïve PAM. For each time point and each animal, two replicates were performed. Three days later cells were fixed and stained and the TCID$_{50}$ was calculated.

**Quantitative reverse transcription-PCR (qRT-PCR)**

To detect viremia, viral RNA was extracted from 140 µL serum with QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA was eluted in 60 µL distilled water and 1 µL was directly used for amplification with the KAPA™ SYBR® FAST One-Step qRT-PCR Kit (Peqlab, Erlangen, Germany) on an ABI 7500 cycler (Applied Biosystems, Foster City, USA). Published primers Pesch PLS (5′-ATGGCCT AGCCAGTCAATC-3′) and Pesch PLR (5′-TCGGCCT AATTGAATAGGTG-3′) were used for amplification [22]. A recombinant cDNA clone of a PRRSV-1 strain was purified using the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany) and spectrophotometrically quantified. The copy number of recombinant plasmids was calculated with the formula: $N = \frac{C \times 185.5 \times 10^{13}}{(factors \ derived \ from \ DNA \ weight, \ volume \ and \ the \ Avogadro \ constant)}$. To obtain a standard curve, a tenfold dilution series of cDNA of the PRRSV-1 clone was included in the qRT-PCR setup. Cycling conditions were 42 °C 5:00, 95 °C 5:00 and 42 cycles of 95 °C 0:03, 60 °C 0:10, 72 °C 0:15 (fluorescence detection step) followed by a dissociation step (95 °C 0:15, 60 °C 1:00, 95 °C 0:15). The numbers of genome copies were calculated with the 7500 System SDS Software (Applied Biosystems, Foster City, USA) based on the standard curve and projected to 1 mL serum by multiplication by 428.4.

To detect viral shedding, nasal swabs were submerged in 2 mL of sterile saline (0.7% NaCl) and thoroughly shaken for 30 s. 200 µL of supernatant were used for viral RNA extraction with the High Pure Viral RNA Kit (Roche Diagnostics, Vienna, Austria) according to the manufacturer’s instructions. RNA was eluted in 50 µL of the kit’s elution buffer and 13.6 µL were directly used for amplification with the LightCycler® RNA Amplification Kit SYBR Green I with a LightCycler® 480-II System (Roche Diagnostics, Vienna, Austria). The nested RT-PCR from Pesch [21] was adapted for detection with SYBR Green I. Reverse transcription and activation was performed according to the manufacturer’s instructions, followed by 32 cycles of 60 °C 0:10, 72 °C 0:22 and 95 °C 0:10. The nested amplification step was carried out with LightCycler® FastStart DNA Master(plus) SYBR Green I (Roche Diagnostics, Vienna, Austria) using 2 µL of the first PCR reaction. Cycling conditions were 25 cycles of 60 °C 0:10,
72 °C 0:20 and 95 °C 0:20. The fluorescence detection step was carried out at 84 °C. The PCR was followed by a dissociation step. Results were considered positive when a clear amplification curve was observed and the melting temperature of the amplicons (calculated by LightCycler® 480 System Software) was within the expected range.

Serology
Serum samples were tested for antibodies against PRRSV with the commercial IDEXX X3 ELISA (IDEXX laboratories, Westbrook, USA) according to the manufacturer’s instructions. Ratios of optical densities of sample and positive control (P/PK ratios) were calculated and considered positive when >0.4.

Statistical analysis
Data were analysed with the SPSS statistics software (SPSS Ltd, Quarry Bay, Hong Kong). One-way ANOVA with Posthoc LSD test was applied for the analysis of all results except for those from the ELISA, which were analysed with the t test with the test value 0. A p value < 0.05 was considered statistically significant.

Results
Virus isolation
Two PRRSV-1 outbreaks in 2013 (farm 883) and 2014 (farm 440) were brought to our attention by the responsible veterinarians. Case 883 was characterized by mild respiratory disorders and a low virus load that persisted over 2 years. In case 440, suckling piglet mortality reached 50%, which is severe for PRRSV outbreaks in Austria, and abortions were observed. Both farms had been found to be PRRSV-1 positive by qRT-PCR before we obtained any samples. To investigate the PRRSV strains responsible for the outbreaks, we isolated two viruses from the serum of diseased piglets after serial inoculation of PAM (termed AUT13-883 and AUT14-440). MARC-145 cells were also susceptible for AUT14-440 without adaption but were not infected by AUT13-883.

Phylogenetic analysis
The sequences of the ORF5 and 7 genes revealed that both isolates belong to PRRSV-1 subtype 1 [7] but the results for ORF5 and ORF7 were not consistent. In the phylogenetic tree based on ORF5, AUT14-440 clustered together with German and South Korean PRRSV-1 isolates (among them KNU-07) (Additional file 1). AUT13-883 represented a different branch of the tree and was most closely related to German strain EU-2a from 1992 [23] and to Austrian strain 2888 [15]. In contrast, when ORF7 sequences were used for phylogenetic analysis, the two Austrian isolates formed a group together with Korean strain KNU-07 [24], a Croatian strain [25] and German strains [26] (Additional file 2).

Since the results of the phylogenetic trees based on ORF5 and ORF7 were inconclusive, complete genome sequences for AUT13-883 and AUT14-440 were determined (GenBank: KT326148 and KT334375). A phylogenetic tree based on the full genome sequences of the isolates presented in this study, 38 published PRRSV-1 strains and PRRSV-2 prototype VR-2332 (Figure 1) confirmed the relationship between the two Austrian isolates, GER09-613 and KNU-07. All four strains cluster together, with no other strain in the same branch. AUT13-883 and GER09-613 are the most closely related strains.

Distinct deletions in the genome of AUT14-440
Assembly of the overlapping sequences resulted in complete genomes consisting of 15022 nucleotides (nt) for AUT14-440 and 15095 nt for GER09-613 and AUT13-883, excluding the 3′ poly(A) tails. Detailed comparison to the European prototype LV revealed marked differences in the region of nsp2. Both Austrian virus isolates and the German strain GER09-613 possess a shorter ORF1a due to deletions in nsp2 (Figure 2). The difference of three nucleotides in ORF1a between AUT13-883 (7188 nt) and LV (7191 nt) results in the deletion of proline 182 in nsp2 (Figure 3A, dotted line box). The deletion is also present in the South Korean strain KNU-07 and has been described for strains from Hong Kong. GER09-613 and AUT14-440 (ORF1a lengths of 7188 and 7152 nt) share a deletion of three nt, resulting in the loss of glutamine 183 in nsp2, one amino acid (aa) downstream of proline 182 (Figure 3A, dotted line box). In comparison to LV, AUT14-440 has further deletions in nsp2 at aa positions 320–323, aa 359–364 and aa 699–700, all in hypervariable regions of nsp2 where deletions are frequently found (Figure 3A, solid line boxes). ORF3 and ORF4 of AUT14-440 were also truncated by 36 nt when compared to LV, resulting in a deletion of 12 aa in the overlapping region of GP3 and GP4 (Figures 3B and C solid line boxes). Shorter deletions (up to 8 aa) in this area have been described for several strains, including HK5 and the Chinese isolate BJEU06-1. AUT14-440 also shows a single nucleotide deletion at position 87 in the 3′ untranslated region (UTR) (Figure 3D, solid line box). All deletions in virus isolate AUT14-440 were also found when RNA from the original serum sample was used for RT-PCR and PCR products were directly sequenced.

Pairwise nucleotide comparison of the complete genome resulted in identities of 87.7, 87.9 and 88.6% with LV for AUT14-440, AUT13-883 and GER09-613. Identities between the isolates were 86.4% (GER09-613...
to AUT14-440), 85.4% (AUT14-440 to AUT13-883) and 89.9% (AUT13-883 to GER09-613) (Figure 2).

**Determination of virulence**

Genetic and phylogenetic analysis showed a striking relationship between the Austrian isolates, the German strain GER09-613 and the South Korean strain KNU-07. The reported severity of the clinical symptoms associated with AUT13-883 and AUT14-440 differed markedly, while the clinical symptoms of GER09-613 have not been described. However, the particular clinical signs in an affected herd provide no more than an indication of the pathogenicity of a PRRSV isolate, as other pathogens may be involved. To assess the virulence and to test Koch’s postulates, an animal trial was performed with the Austrian isolates AUT13-883 and AUT14-440 and the German isolate GER09-613, which clusters in the same branch of the phylogenetic tree.

PRRSV-seronegative pigs were distributed equally into four groups according to their weight and sex. Each
A group of five animals was housed in a separate compartment to avoid cross-contamination. After 1 week of adaptation (day 7 to day 0), pigs from groups GER09-613, AUT13-883 and AUT14-440 were inoculated intranasally with 3 mL medium (1.5 mL in each nostril) containing $1 \times 10^5$ TCID$_{50}$ of the respective strain. Pigs from the control group received the same amount of medium without virus. Clinical signs and rectal temperature were monitored daily. A scoring system was applied that rates liveliness, dyspnoea, coughing, nasal and ocular discharge, conjunctivitis and cyanosis with scores from 0 (physiological) to 3 (severe clinical signs). Daily scores for each animal were added to obtain a value for the overall health status and the mean clinical scores for the four groups were calculated (Figure 4A).

All pigs infected with AUT14-440 showed mild dyspnoea on three or more study days. Other clinical signs that occurred frequently in all animals in this group were apathy and conjunctivitis. Two waves of clinical signs could be distinguished: the first wave occurred around day 4 post infection (pi) and the second, with more prominent signs of illness, took place around day 12 pi. In the other infection groups, only one (AUT13-883) or two (GER09-613) animals showed mild dyspnoea for no more than 2 days in a row. Pigs in the control group showed no relevant clinical signs in the trial, except for animal #4 that had to be euthanized on study day 10 due to a severe lameness in the hind legs. There was no significant difference in rectal temperature between the four groups.

The pigs were weighed at -7, 0, 3, 7, 10 and 13 days post infection (dpi). For each week of the trial (study days -7 to 0, 0 to 7, 7 to 13) daily weight gain was calculated and is shown for each group as a box plot with maximum and minimum values represented as whiskers (Figure 4B). From the first week to the second week of the trial, all groups showed an average increase of daily weight gain between 0.08 and 0.16 kg. From the second to the third week, the control group and group GER09-613 showed a mean increase of daily weight gain of 0.05 and 0.11 kg, whereas in the groups infected with the Austrian field isolates daily weight gain did not increase (mean values of -0.07 kg for AUT14-440 and -0.02 kg for AUT13-883). Despite the apparent differences between the groups, the figures are not statistically significant due to the small group size and the high variation within the groups.

**Figure 2** Detailed comparison of complete genomes of GER09-613, AUT14-440, AUT13-883 and Lelystad virus (LV) (table) and schematic overview of nucleotide differences between LV and the isolates presented in this study. Black bars represent areas with a low degree of similarity to LV. Open reading frames (ORF) of PRRSV that encode non-structural proteins (blue) and structural proteins (orange) are shown.
Multiple alignments of partial nsp2 (A), GP3 (B) and GP4 (C) amino-acid sequences of eleven different European PRRSV strains.

Only aa differing from Lelystad virus (LV) are shown, with identical aa represented by dots. Deletions compared to LV are marked in red. Deletions of AUT14-440 are highlighted with solid boxes; a deletion in all three isolates is indicated with a dotted box. The numbers above the alignment indicate the position in the protein. A single aa deletion is present in all three isolates and KNU-07 at position 182 or 183 in nsp2. In AUT14-440 aa 320–323, aa 359–364 and aa 699–700 are deleted compared to LV. The box marks a 12 aa deletion in the overlapping region of GP3 and GP4 of AUT14-440. Multiple alignments of different PRRSV strains show that different nucleotides are present in the Lelystad virus (LV) genome, with identical nucleotides represented by dots. A deletion of 1 nt at position 87 in AUT14-440 is marked in red and highlighted with a solid box.
groups ($p = 0.055$ between GER09-613 and AUT14-440 for the third week).

**Gross pathology and histopathology**

At 13 and 14 dpi, pigs were euthanized and necropsied with special emphasis on the lungs. No other organs than lungs showed pathological lesions. Samples from each lung lobe were fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin and examined histologically. Lesions were hypertrophy and hyperplasia of pneumocytes, septal infiltration by mononuclear cells, perivascular and intraalveolar accumulation of inflammatory cells and necrotic debris. Depending on their occurrence in different lung lobes and on the severity of the lesions, a total score per lung was calculated, applying a PRRSV specific scoring system that has been described elsewhere [27]. The mean values for the Austrian isolates were 52.4 ± 20.9 for AUT14-440 and 53.6 ± 9.4 for AUT13-883 (Figure 5A). In these two groups all types of lesions mentioned above were present in mild to severe manifestations with no visible differences between the groups. The results differed significantly ($p < 0.01$) from both the control group with a mean score of 5.6 ± 3.0 and group GER09-613, which mainly showed minor accumulations of inflammatory cells (mean histological score of

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**Figure 4 Clinical signs and daily weight gain.** A: Pigs were examined daily and a clinical score was calculated based on the severity of PRRSV-associated clinical signs. The mean values for each group are shown with a continuous line and single spots represent individual animals. As group AUT14-440 was euthanized on day 14 pi there are values for 1 day more than for the other groups. B: Pigs were weighed at six time points and daily weight gain was calculated for three time frames: 1st week (−7 to 0 dpi), 2nd week (0 to 7 dpi) and 3rd week (7 to 13 dpi) of the trial. Whiskers represent maximum and minimum values. Differences between the groups were visible for the last time period, 7–13 dpi, although they are not statistically significant ($p = 0.055$ between GER09-613 and AUT14-440).
Viremia and virus shedding

To exclude the possibility of cross-contamination between the groups, 50 µL of serum taken 3 dpi from one animal per group (#10, 14, 19) were inoculated onto naïve PAM and virus was re-isolated. RT-PCR and subsequent sequencing of the 700 bp PCR amplicon covering ORF4 confirmed the identity of the viral strains. Compared to the parental strain, two point mutations were detected in each strain. One of them was the same for all three strains and resulted in an amino-acid change from aspartic acid to asparagine at position 33 in GP4. In addition melting temperatures of amplicons from positive nasal swabs confirmed that no cross-contamination had occurred (data not shown).

Viral nucleic acids in serum could be detected with qRT-PCR as early as day 3 pi in all animals from group AUT14-440 and in two animals from group AUT13-883 (Figure 6). In the GER09-613 group, the first virus-positive animals were detected 10 dpi. On day 13 pi all animals from this group except one (#8) tested positive for viral RNA; animal #8 remained negative for qRT-PCR as well as for titration on PAM (data not shown) on all sampling days. The number of genomic copies in the serum of animals infected with AUT14-440 declined towards the end of the trial. In the AUT13-883 group, the onset of viremia differed between the pigs. The maximum number of genome copies per mL serum was up to 100 times higher in the AUT14-440 group than in the other groups. On days 3 and 7 pi, the difference between group AUT14-440 and all other groups was significant (p < 0.01), whereas on day 10 pi only the difference to the control group and the AUT13-883 group was significant (p < 0.05). Serum titres on PAM were also highest in the AUT14-440 group (up to 2.3 × 10³ TCID₅₀/mL, data not shown). Viral RNA or infectious virus could be detected in the serum of all infected animals, except for #8, with qRT-PCR and titration on PAM on one or more sampling days. The animals from the control group gave negative results with both assays throughout the trial.

To examine virus shedding from the upper respiratory tract, nasal swabs were taken at −7, 3, 7, 10 and 13 dpi and analysed with qRT-PCR for PRRSV-specific nucleic acids (data not shown). All animals from infected groups were virus-positive 3 dpi. Group GER09-613 tested completely negative on day 7 pi and one pig (#8) remained negative until the end of the trial, while the other animals tested positive on day 10 and 13 pi. Virus shedding could be determined for four pigs each from groups AUT14-440 and AUT13-883 on study days 7 and 10, whereas only two animals from group AUT14-440 and all animals from group AUT13-883 were positive on the last

![Figure 5 Histological lung lesions.](image)

For each animal, a histopathological lung lesion score was calculated by considering the degree of PRRSV-associated lesions in every lung lobe. Histological lesion scores are presented in a table and as a box-plot. Mean score ± standard deviation and individual scores for each animal are displayed for all groups. Box-whiskers represent maximum and minimum values. Letters indicate significant differences (p < 0.01) from control group (a), group GER09-613 (b), group AUT14-440 (c) and group AUT13-883 (d).
sampling day, day 13. The nasal swabs from the control group tested negative for PRRSV-specific nucleic acids at every time point.

**Serology**

Antibody titres against PRRSV were determined at the beginning of the trial to ensure the PRRSV-free status of the animals. At this time point all pigs tested negative for N-specific antibodies with the commercial IDEXX X3 ELISA (Figure 7). Pigs were further screened for seroconversion 7, 10 and 13 dpi. First detection of PRRSV-specific antibodies was possible 7 dpi in the sera of two pigs from groups AUT14-440 and GER09-613. On days 10 and 13 pi all infected animals but one (#19) tested positive in ELISA. The clearest antibody response with the highest P/PK ratios was seen in the AUT14-440 group. The animals from the control group were negative for PRRSV-specific antibodies at every time point.

**Discussion**

Austria is surrounded by seven neighbouring countries and lies on the border between Western and Eastern Europe. Due to its central location it has become a major European corridor for travel and the movement of goods, including live pigs. In fact, one of the isolates (AUT440-14) originated from a farm directly adjacent to the major west-east transit road. The reintroduction of PRRSV into virus-free herds is the most urgent yet least understood problem for the swine industry in Austria. Epidemiological evidence is often insufficient as only few ORF5 and ORF7 sequences from Austria are accessible at GenBank, most of them originating from only three publications [15–17]. In none of these cases were sequence data correlated to the biological properties of the isolates.

In this study, the initial question about the phylogenetic background of two field strains not only led to complete genome sequences of two recent Austrian field isolates but also to their characterization in vivo. (Phylo-) genetic information could be interpreted in the light of the phenotypic properties of the isolates. This is especially important because genetic relationships do not give evidence for the virulence of PRRSV strains [6]. To go beyond the descriptive determination of selected sequences, it is necessary to address the pathogen’s properties by carefully characterizing the isolate.

The initial virus isolation in this study was performed on primary PAM, which are believed to be the main target cells of PRRSV in vivo and are widely used for PRRSV-1 isolation and propagation [28]. The much more convenient simian cell line MARC-145 can usually not be used for unadapted PRRSV-1 virus strains, although it is readily infected by PRRSV-2 without adaptation [6, 28]. Interestingly, it also supported growth of the first passage
of AUT14-440, a PRRSV-1 isolate. This observation encouraged us to investigate the phylogenetic classification of the strain.

Virus isolation is rarely performed in routine PRRSV diagnostics and sequences are normally obtained directly from clinical samples. Generally, only ORF5 or ORF7 sequences are determined. However, there are doubts about the validity of phylogenetic trees based solely on ORF5 or ORF7 sequences because of the recombination potential and high rate of nucleotide substitution shown by these genes [6, 14]. As a matter of fact, in the phylogenetic trees we determined based on ORF5 and 7 the relationship between the two Austrian isolates and closely related strains remained unclear as AUT13-883 grouped differently in the two trees. The difference might relate to recombination events, which are believed to have a major impact on PRRSV diversity [6, 29]. No evidence for this phenomenon was found by recombination analysis, probably due to the small number of full PRRSV-1 genomes available in GenBank (data not shown). This finding underlines the need for more full-length genome sequencing. The phylogenetic tree based on full genomes clearly showed relatedness between the two Austrian isolates, the German strain GER09-613 and the South Korean isolate KNU-07. However, it has to be considered that the four strains cluster together in a phylogenetic tree despite not being closely related (identities between 85.4 and 90.2%) and that only 38 PRRSV-1 strains were available for comparison, most of them from earlier than 2010. The picture might be less clear if the full genome sequences of more (recent) strains were available for inclusion in the analysis. At present we can only speculate whether the South Korean strain was imported to Europe or whether the cluster is the result of convergent evolution. It is also possible that all four strains originate from older strains from Germany (e.g., BH_95_10-08_EU) that show close relationships in ORF5 and ORF7 but for which complete sequences are not available. Detailed epidemiological statements will not be possible without a broader range of full-length PRRSV genomes.

Nevertheless, comparing not only a small part of the 15 kb PRRSV genome but full sequences revealed remarkable characteristics of the strains and provided indications that the four strains have a common origin. AUT13-883 and KNU-07 have a proline deletion in nsp2 that has only been noted in several strains from Hong Kong [30]. AUT14-440 and GER09-613 also share a single aa deletion in nsp2, a single position after the proline. Additionally, on either side of the 12 aa deletion in GP3 in AUT14-440 the four strains share areas of aa sequences that are very similar to one another but clearly differ from those of other PRRSV-1 strains, providing further indications of a possible relationship.

A closer look at the full genome sequences revealed additional deletions in strain AUT14-440. In nsp2, three more deletions of 2–6 aa support the conclusion from the phylogenetic analysis that the isolate is not as closely related as the three other strains. The 12 aa deletion in the overlapping region of GP3 and GP4 is unique to this strain. Other published isolates that carry a deletion in this highly variable area only miss 1–8 aa compared to LV and the concomitant occurrence of deletions in nsp2 and ORF3/4 has only been described for a few Chinese strains [31–33]. The first strains carrying a deletion in the overlapping region of ORF3 and ORF4 were isolated in Denmark as long ago as 1992, shortly after the discovery of PRRSV. In subsequent evaluations, deletion mutants were reported to evolve more quickly than non-deleted viruses [34]. This might be due to immunological pressure, since neutralizing antibodies against this region in GP4 have been described in vitro for LV [35] and two Belgian strains [36]. This region and the corresponding region in GP3 were also shown to be immunogenic for other isolates, including a Danish deletion mutant with an 8 aa deletion [34, 37]. Therefore, it is very likely that the highly variable region in the overlap of GP3 and GP4 is under negative selection pressure, causing deletions or mutations to occur [38]. This is in line with the existence of very similar aa sequences next to the deletion in the Austrian isolates and their nearest relatives GER09-613 and KNU-07, indicating that the four strains evolved in a similar direction, probably due to immunological pressure. The 12 aa deletion in AUT14-440 might prevent the binding of GP4-specific antibodies and thereby result in an evolutionary advantage. This idea will be tested in further experiments.

Another interesting aspect of the deletion is the fact that the heterotrimeric minor glycoproteins GP2, GP3 and GP4 are abundant on the viral surface and probably determine cell tropism [39]. A deletion of 12 aa in two of these proteins might lead to an altered cell tropism, as seen in this study for AUT14-440, which is, despite its type 1 genotype, capable of infecting MARC-145 cells. The single nt deletion in the 3′UTR of AUT14-440 has not been described in other PRRSV-1 strains, although there is a report of a deletion at a different position within the 3′UTR in highly pathogenic Chinese strains [40]. As the 3′UTR is essential for PRRSV replication [41], deletions in this area might have an implication for the replication efficacy of the virus.

AUT14-440 differed from the other Austrian isolate AUT13-883 and the German isolate GER09-613 also in other aspects. It was the only strain to cause clear respiratory distress in pigs in the animal trial. Animals infected with AUT14-440 ceased to increase daily weight gain in the second week after infection,
highlighting the economic relevance of the strain. Although pigs from the other groups remained relatively healthy throughout the trial, moderate to severe lung lesions were found in pigs infected with AUT13-883, which also showed varying developments of daily weight gain. It is conceivable that clinical signs would have been more prominent in all infection groups if a less natural route of infection, e.g., intramuscular, or higher viral titres had been chosen.

Viremia was already detected 3 dpi in pigs infected with the Austrian strains, which is in accordance with other experimental infections [42, 43], whereas the GER09-613 group showed the first viral RNA and titres in serum 10 dpi. A likely explanation for the late onset and the lack of clinical signs is that strain GER09-613 was cell-culture adapted on MARC-145 cells and hence potentially attenuated and thus had to re-adapt to the host. Future studies will be necessary to address the sequence differences between inoculated GER09-613 and the re-isolated virus.

In the present study, high amounts of viral RNA in blood were associated with severity of clinical signs, with titres of AUT14-440 exceeding those of the other strains by up to 100-fold. In contrast, the amounts of viral RNA in nasal swabs were lowest for pigs infected with AUT14-440, indicating that the amount of virus shedding via the respiratory route is not necessarily correlated with viremia. The detection of virus shedding 3 dpi is probably an artefact of intranasal infection, as pigs from group GER09-613 do not show any viral RNA in nasal swabs 7 dpi, which corresponds to the late onset of viremia in this group.

The early detection of non-neutralizing N-specific antibodies is in accordance with previous studies [42, 43]. The IDEXX X3 ELISA is not quantitative but the high P/PK ratios of group AUT14-440 are in agreement with the high number of viral genome copies in the blood of animals infected with this strain.

In summary, we have determined the complete genome sequence of two recent Austrian field isolates of PRRSV and interpret the nucleic acid sequences and the phylogeny in the light of the strains’ in vivo characteristics. Both isolates cluster with a German field isolate from 2009 and the South Korean KNU-07 strain in whole genome phylogenetic analysis. One of the field isolates, AUT14-440, caused clinical signs in an animal experiment, grown on MARC-145 cells and showed exceptional deletions. These well-characterized isolates represent an excellent basis for further studies on the implications of the described molecular properties on virus entry, replication and pathogenicity. It remains unclear whether the similarities to East Asian strains are unique to European strains because of the lack of full genome sequences of strains currently present in the field. As PRRSV strains are subject to constant evolutionary pressure, both from vaccination and from instruments of modern herd management, it is of the utmost importance to keep up with the increasing diversity of PRRSV by studying current isolates.

Additional files

Additional file 1. Phylogenetic analysis of ORF5. Phylogenetic tree based on ORF5 nucleotide sequences of 41 PRRSV-1 strains and PRRSV-2 prototype VR2332 as an out-group as well as the two closest related sequences in NCBI BLASTn phylogenetic tree for each isolate. The PRRSV strains presented in this study are marked in red. The tree was constructed using the neighbour joining method with the numbers at the nodes representing bootstrap values in % of 1000 replicates. Scale bar: number of substitutions per site.

Additional file 2. Phylogenetic analysis of ORF7. Phylogenetic tree based on ORF7 nucleotide sequences of 41 PRRSV-1 strains and PRRSV-2 prototype VR2332 as an out-group as well as the two closest related sequences in NCBI BLASTn phylogenetic tree for each isolate. The PRRSV strains presented in this study are marked in red. The tree was constructed using the neighbour joining method with the numbers at the nodes representing bootstrap values in % of 1000 replicates. Scale bar: number of substitutions per site.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

LJS, HK, BL, CR, IH-P and TR planned and conceived the experiments. LJS, LZ and BL performed all other laboratory work and analysed the data. LJS, BL and TR interpreted the results and designed the figures. LJS and TR wrote the manuscript. All authors read and approved the manuscript.

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References


3.2 Emergence of a virulent porcine reproductive and respiratory syndrome virus (PRRSV) 1 strain in Lower Austria
Emergence of a virulent porcine reproductive and respiratory syndrome virus (PRRSV) 1 strain in Lower Austria

Leonie J Sinn1*, Eva Klingler2, Benjamin Lamp1, Rene Brunthaler3, Herbert Weissenböck3, Till Rümenapf1 and Andrea Ladinig4*

Abstract

Background: In spring 2015, an outbreak of porcine reproductive and respiratory syndrome (PRRS) struck Lower Austria caused by a PRRS virus (PRRSV) strain spreading rapidly among both previously PRRSV negative and vaccinated pig herds. This case report describes the first well-documented emergence of the PRRSV strain responsible for this outbreak.

Case presentation: A PRRSV seronegative piglet-producing farm in Lower Austria encountered losses in foetuses and suckling piglets of up to 90%; clinical signs in sows and nursery piglets included fever and reduced feed intake. Additionally, high percentages of repeat breeders and losses of up to 40% in nursery piglets occurred. An infection with PRRSV was suggested by the detection of antibodies by enzyme linked immunosorbent assay and confirmed by quantitative real time PCR. The underlying PRRSV strain, termed AUT15-33, was isolated by passage on porcine alveolar macrophages, partially sequenced (ORF2-7) and grouped as PRRSV-1, subtype 1. In phylogenetic analysis of the genome region coding for the structural proteins, ORF2-7, AUT15-33 clustered with Belgian strains but identities were as low as 88%. In contrast, analysis of ORF7 sequences revealed a close relationship to Croatian strains from 2012 with an identity of 94–95%.

Conclusions: In the year following the outbreak, the same PRRSV strain was identified repeatedly in different regions of Austria. It can be speculated that the new strain has novel advantageous properties.

Keywords: Porcine reproductive and respiratory syndrome virus (PRRSV), Austria, Field isolate, Acute outbreak

Background

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically important viruses affecting the global swine industry. PRRSV is a small, enveloped virus with a single-stranded RNA genome of positive polarity, which is grouped in the family Arteriviridae [1–3]. Due to the high degree of genetic diversity, PRRSV was recently divided into two species: PRRSV-1 (formerly European genotype 1) and PRRSV-2 (formerly North American genotype 2) [4, 5]. Extensive genetic differences do exist not only between but also within the two species leading to the sub-classification into at least three European subtypes [6]. Depending on the strain great differences exist, for example in the ability of propagation in different cell lines in vitro [1, 7] or in the pathogenicity in vivo [8–10].

Clinical presentation of PRRS varies greatly between herds and is influenced by genetic and virulence differences of PRRSV isolates, host immune status, host susceptibility, concurrent infections and other management factors [11]. Typical clinical signs of PRRS in nursery and grow/finishing pigs include respiratory signs and reduced growth performance [1, 12]. Of particular importance are secondary and concomitant infections since PRRSV was shown to have an additive or synergistic
effect with other bacteria and viruses [13–15]. Reproductive disease associated with PRRSV is characterized by abortions, early farrowings, foetal death and the birth of weak, congenitally infected piglets resulting in elevated pre-weaning mortality [16–18]. Highly pathogenic PRRSV strains have been described for both PRRSV-1 (strain Lena, subtype 3 [19]) and PRRSV-2 (atypical PRRS caused by strains with a characteristic deletion in nonstructural protein 2 [20]). They are characterized by high fever and high mortality rates in pigs of all age groups.

Diagnostic methods include e.g., virus isolation, histologic staining techniques and reverse transcription polymerase chain reaction (RT-PCR), which is most commonly used for routine diagnostics. For virus isolation PRRSV can be grown on primary porcine alveolar macrophages (PAM), which are obtained from lungs of PRRSV-free pigs. The only PRRSV-permissive cell lines, MA-104 or its clone MARC-145, rarely support the spontaneous growth of PRRSV-1 strains. The detection of PRRSV-specific antibodies is most commonly performed by enzyme-linked immunosorbent assay (ELISA). Control measures for PRRSV include the prevention of virus introduction into herds by applying strict biosecurity standards and the regular use of modified live vaccines (MLV).

Austria is a small country in Central Europe that directly neighbours eight nations. Due to the geopolitical situation, Austria is bridging the trade of western and eastern, northern and southern European countries. Import or transit of animals or animal products implies the risk of acquiring virus diseases of livestock and hence Austria might act as a sentinel. PRRSV is considered endemic in Austria (as in most other countries) although detailed epidemiological data is limited [21, 22]. Available sequence information on Austrian PRRSV strains include several ORF5 and ORF7 sequences and two full-length genomes of PRRSV isolates [23–26], all belonging to PRRSV-1 subtype 1.

Here we describe the first well-documented case of a cluster of acute outbreaks of PRRS in Lower Austria in spring 2015. Losses on the presented piglet-producing farm in Lower Austria went up to 90 % in one farrowing batch. The underlying virus strain, named AUT15-33, showed a high similarity with Croatian strains in ORF7 and proved its epidemic potential in the year following the outbreak by spreading to other regions in Austria.

Case presentation
Anamnestic and physical findings
The case herd was kept on a family owned, piglet producing farm located in Lower Austria, which was known to be free of PRRSV since ten years based on routine serological testing of sows and nursery piglets performed twice per year. The farm was producing piglets with 80 sows in a 3-week batch-farrowing interval; suckling period was about 28 days. At the end of the nursery period, 30 kg piglets were sold to one finishing farm. Gilts were bought from one multiplier herd in Lower Austria.

Clinical problems in the herd started in the beginning of April 2015; sows had reduced to completely absent feed intake and high fever (>41 °C). Additionally, cyanosis on ears and tail was visible in individual sows, which developed into ear and tail necrosis later in the course of disease (Fig. 1a). The herd veterinarian decided to treat the sows with acetylsalicylic acid due to the suspected diagnosis of an influenza virus infection. However, clinical signs did not improve and reproductive disorders started to occur when the next batch of sows farrowed mid-April. In the particular farrowing batch, around 50 % of piglets were born dead and another 40 % of piglets died within the first days of live. Only eleven piglets were weaned from nine sows and all showed reduced growth performance (Fig. 1a). Two sows farrowed

Fig. 1 (a + b) Clinical signs in sows (a) and nursery piglets (b). a Affected sows showed cyanosis on ears and tail (green arrows) and gave birth to dead or weak piglets. b Piglets in nursery suffered from various clinical signs including swollen joints, stomach ulcers/gastritis, pericarditis and pneumonia.
ten days delayed and were less severely affected; a total of 20 piglets could be weaned from those two sows, with five piglets clearly retarded in growth. A similar clinical picture was observed in the next farrowing batch: 38 piglets from ten litters survived the first two weeks of life but were clearly reduced in their growth performance. Interestingly, all sows farrowed at their due date with the exception of one sow which farrowed two days early and delivered dead piglets only.

Reproductive problems also occurred in early gestation leading to a return to oestrus rate of 60 % in sows bred beginning of April and 40 % in sows bred end of April.

Clinical problems in the nursery started end of April in piglets about five weeks of age (one week after weaning). Piglets showed fever, swollen joints and lameness, severely reduced feed intake and runting (Fig. 1b). About 40 % of piglets from this age group died or had to be euthanized. Antibiotic treatment with various antimicrobials (including amoxicillin, colistin sulphate, ceftiofur, etc.) did not improve the situation. Clinical signs spread slowly throughout the nursery. Next, the oldest group of piglets close to delivery to the finishing farm was affected. Piglets showed signs of diarrhoea, reduced growth performance, anaemia and about 10 % of piglets died in this age group. The least affected group of piglets was the age group in mid nursery; here piglets showed mild respiratory signs like coughing and dyspnoea, but no losses occurred.

A summary of clinical observations including a time course of events is illustrated in Fig. 2.

Diagnostic methods and laboratory findings
First samples for diagnostics were taken end of April from sows, which started to show clinical signs at least two weeks prior to sampling. Blood samples were taken from nine sows for serological investigation. Antibodies against PRRSV were detected by ELISA in sera of all sows. To investigate concomitant infections in nursery piglets, two piglets from the most severely affected age group (around seven weeks old at the time of submission), showing poor body condition, enlarged inguinal lymph nodes and respiratory signs like coughing were selected by the herd veterinarian and submitted for necropsy and further diagnostics to the University of Veterinary Medicine Vienna. Post mortem investigations found poor retraction of the lung and consolidation of the cranio-ventral areas. A catarrhal enterocolitis was diagnosed in one piglet. Histologically, atelectasis with intralobular, interstitial pneumonia including hyperplasia of type II pneumocytes, as well as a catarrhalic to purulent bronchopneumonia were seen in the lungs of both pigs (Fig. 3). Bacterial isolation was only performed on organs showing pathological alterations (intestines and lung). While in intestinal samples no pathogenic bacteria could be detected, *Staphylococcus hyicus* could be isolated from the lung of both pigs by conventional bacteriological culture. To exclude an involvement of PCV2 an in situ hybridization (ISH) on inguinal lymph nodes was performed. No histologic lesions were found in the lymph nodes and no PCV2 was detected by ISH.

Due to the wet summer of 2014 elevated mycotoxin levels were expected for corn fed in 2015. Mycotoxin analyses revealed increased levels of deoxynivalenol (DON, 5040 µg/kg) and zearalenone (ZEA, 851 µg/kg). The proportion of corn in feed was about 16.5 % for sows and 30 % for piglets.

Since the clinical course of infection was unusually severe for Austrian conditions and reports about PRRS outbreaks in the same region accumulated, the PRRSV strain was further characterized. Blood samples were collected from nine nursery piglets showing acute clinical signs like fever and reduced alertness in order to isolate the virus and perform RT-PCR and sequencing. To this end naïve primary cells (PAM) and the cell line
MARC-145 were inoculated with serum from each piglet. A PRRSV-specific immunofluorescence staining of PAM (Fig. 4) but not of MARC-145 cells could be detected after two days. This finding was confirmed after passaging the supernatant on new PAM and MARC-145 cells (data not shown). In accordance, all nine serum samples were tested highly positive when using a commercial PRRSV quantitative RT-PCR (qRT-PCR) Kit with genome copy numbers between $1.4 \times 10^8$ and $4.3 \times 10^9$ (Table 1). In conclusion, a new PRRSV strain, named AUT15-33, could be isolated from serum of piglets showing acute illness on primary PAM.

Total RNA was extracted from PAM five days after inoculation and used for RT-PCR. Two primer pairs (PRRSV-I-F 5’-GACCATATCTGCAACCTGAGAC-3’/PRRSV-I-R 5’-CAATTTGTGAAGACATCTCATC-3’ and PRRSV-II-F 5’-CTTTTCTACGCCTGAGAAATGAG-3’/PRRSV-II-R 5’-TTTGGATCCAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’) were applied to generate overlapping PCR amplicons, which covered the whole genome region encoding the structural proteins (ORF2-7).

The DNA fragments were subjected to gel electrophoresis, purified and sequenced by a commercial laboratory (Eurofins Genomics, Ebersberg, Germany). The determined ORF2-7 genome sequence of AUT15-33 was submitted to GenBank (KU494019).

Initial phylogenetic analysis and identity calculations were carried out with NCBI’s Basic Local Alignment Search Tool (BLAST) and confirmed by NCBI’s Conserved Domain Database (CDD). The results showed that the new PRRSV strain AUT15-33 shares similarities with other PRRSV strains isolated from Europe and Asia, but has unique genetic characteristics that differentiate it from the previously described strains.

### Table 1: qPCR Results

<table>
<thead>
<tr>
<th>Control</th>
<th>#33</th>
<th>#38</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR result (genome copies/mL)</td>
<td>-</td>
<td>$2.3 \times 10^9$</td>
</tr>
<tr>
<td>Brightfield</td>
<td><img src="500%CE%BCm" alt="control" /></td>
<td><img src="500%CE%BCm" alt="#33" /></td>
</tr>
<tr>
<td>Cy3 fluorescence staining with anti-N-mAb</td>
<td><img src="500%CE%BCm" alt="control" /></td>
<td><img src="500%CE%BCm" alt="#33" /></td>
</tr>
</tbody>
</table>
Search Tool for nucleotides (BLASTn). All PRRSV-1 strains with full ORF2-7 sequences deposited in GenBank as well as the two closest neighbours - as determined by NCBI BLASTn - were used to construct the phylogenetic trees. For ORF5 and ORF7, a nucleotide identity >99% was found with the Austrian PRRSV strain ‘Acro’ (KT265737), which has been submitted to GenBank in July 2015 and very likely originates from the same PRRS outbreak. In the phylogenetic tree based on ORF7 both AUT15-33 and Acro cluster with several Croatian strains from 2012 [27], one of them (CRO_PRRSV_3, KF498723) is shown as a representative, but not with other current Austrian PRRSV sequences from 2013 and 2014 [26] (Fig. 5). The identity with the Croatian strains ranges from 94 to 95%. Due to missing sequence data for the Croatian strains, the relatedness to them could not be investigated further. The phylogenetic tree built with ORF5 sequences shows the new Austrian isolate clustering with the Belgian

Table 1 Viral load in serum of acutely affected nursery piglets quantified by a commercial PRRSV qRT-PCR Kit (TagMan®NA and EU PRRSV Reagents, Ambion, Carlsbad, USA)

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>CT-value</th>
<th>PRRSV RNA copies/mL serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>16.02</td>
<td>2.2 x 10^9</td>
</tr>
<tr>
<td>33</td>
<td>15.99</td>
<td>2.3 x 10^9</td>
</tr>
<tr>
<td>34</td>
<td>18.41</td>
<td>4.8 x 10^8</td>
</tr>
<tr>
<td>35</td>
<td>16.02</td>
<td>2.2 x 10^9</td>
</tr>
<tr>
<td>36</td>
<td>17.26</td>
<td>1.1 x 10^9</td>
</tr>
<tr>
<td>37</td>
<td>14.99</td>
<td>4.3 x 10^9</td>
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<td>38</td>
<td>20.39</td>
<td>1.4 x 10^8</td>
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<td>39</td>
<td>18.98</td>
<td>3.3 x 10^8</td>
</tr>
<tr>
<td>40</td>
<td>17.51</td>
<td>8.6 x 10^8</td>
</tr>
</tbody>
</table>

Fig. 5 Phylogenetic analysis based on ORF7 nucleotide sequences of 54 PRRSV-1 strains and PRRSV-2 prototype VR2332 as an out-group. The PRRSV strain presented in this study is marked with a solid box and the associated sub-tree is highlighted with a dotted box. The tree was constructed with the software CLC Sequence Viewer 7.6 (CLCBIO, Aarhus, Denmark) using the neighbour joining method with the numbers at the nodes representing bootstrap values in % of 1000 replicates. Scale bar: number of substitutions per site.
strains 13 V091 and 08 V194 [28, 29] (Fig. 6). However, the nucleotide identity between these and AUT15-33 is only 86 %, the same as with the PRRSV-1 prototype strain Lelystad virus (LV). A similar pattern is seen in a phylogenetic tree based on a larger sequence part (3.2 kb) including the genome region coding for the structural proteins (ORF2-7) (Fig. 7). Here the identity of AUT15-33 to both Belgian strains mentioned above and to LV is 88 %.

Further steps and outcome of case

When laboratory tests confirmed an infection with PRRSV, the herd veterinarian decided to perform mass vaccination of all pigs on site using a PRRSV-1 MLV (Porcins’ PRRS, MSD Animal Health, Boxmeer, Netherlands). Pigs were vaccinated twice: the first vaccination was performed in the first week of May, the second vaccination four weeks later. Additionally, the mycotoxin content in feed was reduced by lowering the percentage of corn within the diet.

The clinical situation in sows improved during the course of May; about 88 % of sows inseminated mid-May became pregnant. With the exception of two sows, which delivered dead piglets only, no further reproductive problems occurred in the group of sows farrowing end of May. In nursery piglets an improvement of clinical signs was not seen until end of May in the most severely affected group of piglets. The age group at mid-nursery never experienced severe clinical signs and no losses occurred in this group.

The route of PRRSV introduction into the case herd could not be identified. Gilts were unlikely the source of PRRSV introduction because a detailed screening for PRRSV antibodies in the multiplier herd was negative and no other clients of that herd experienced problems.
with PRRSV. Since the case herd shared boars with a commercial piglet producing farm, which experienced an outbreak with PRRSV, this was the most likely route of PRRSV introduction into the case herd. Also, manure-pumping equipment was shared between the case herd and other farms. Manure was pumped from the case herd about two weeks prior to the outbreak and the equipment could have been a potential route of PRRSV introduction. Because several farms in the surrounding area experienced PRRS outbreaks at the same time, other routes of infection like aerosol transmission, people or vehicles acting as vectors for the virus cannot be excluded.

Ever since the first outbreaks with PRRSV AUT15-33 have been identified, the particular virus strain has been found in various regions of Austria. The intramural diagnostic laboratory alone could confirm the same strain on 16 additional farms. More recently, similar PRRSV isolates were found in pig farms in Germany (AL, personal communication).

**Conclusions**

The acute outbreak of PRRS in the case herd was characterized by massive losses in both suckling and nursery piglets, indicating a PRRS outbreak rather unusual for Austria with high economic losses. This is of particular importance since it is the first well-documented case of a cluster of outbreaks in Lower Austria in 2015 most likely caused by the same virus strain.

Before the PRRS outbreak, despite the high levels of mycotoxins in the feed, performance of sows and piglets in the case herd was good with losses below 2% and minimal antimicrobial treatment in nursery piglets. The
severe clinical signs and high losses in nursery pigs suggest that PRRSV is important as a primary pathogen as well as an immunomodulating factor causing secondary infections in pigs (e.g., *Staphylococcus hyicus* in this case).

As an immediate intervention strategy, the herd veterinarian decided to perform mass vaccination with MLV. On the other hand, PRRSV MLVs were proven to be able to reduce clinical signs and lesions after PRRSV infection [30]. On the other hand, PRRSV MLVs were reduced wild-type virus shedding from infected populations to the environment [36]. Also, MLV could be effectively used to eliminate PRRSV from herds by homogenizing the immune status of the animals since it was known that PRRSV does not persist in immune populations [37, 38]. Considering the slow spread of the virus within the case herd, the use of MLV might have been beneficial for pigs that have not yet been naturally infected.

The isolation of PRRSV field strains in cell culture is an important tool to further characterize virus strains because virus growth in cell culture allows sequencing of larger genomic regions. For phylogenetic analysis and recombination studies, both crucial to monitor PRRSV epidemiology and to evaluate the effectiveness of current vaccine strategies, it is essential to sequence parts of the genome larger than ORF5 or ORF7. These regions have been widely used in routine diagnostics but account for only 4 and 2.5 % of the whole genome, which limits their significance for phylogenetic studies, especially if only one of them is determined. ORF7 of AUT15-33 shows a high degree of similarity to Croatian strains from 2012, a finding that cannot be confirmed in analysis of larger genomic regions because for these strains only ORF7 sequences exist (J. Prpić, personal communication) [36]. When analysing ORF2-7 or ORF5, AUT15-33 clustered with strains from Belgium but identities were not higher than 88 %, excluding a close relationship. The same identity occurs to the prototype strain LV, which clusters totally different in a phylogenetic tree. The reason is that phylogenetic analysis is not based on identity calculations but on algorithms calculating evolutionary development. Sequences that cluster in the same lineage and show a high sequence identity to AUT15-33 could fill the evolutionary gap between the Belgian strains and AUT15-33 but are missing. Surprisingly there is no close relationship to Austrian strains previously detected (e.g., to strains from 2013 to 2014 [26] or earlier [24, 25]). It is questionable whether the lack of close relatives is due to the few sequences available or to a novel introduction to Austria. We favour the idea that the strain was newly introduced to Austria because the outbreak of AUT15-33 occurred simultaneously on many farms and caused high losses, indicating naïve herds. With regard to the high similarity to the Croatian strains an origin in south-eastern Europe seems possible but as long as there is limited data available on PRRSV strains in Austria and neighbouring countries, it can only be speculated about potential introduction routes of AUT15-33.

Availability of sequence information from strains currently circulating in the field is not only important for epidemiology but also for evaluation of diagnostic methods. Since PRRSV is a RNA virus and therefore has a high mutation frequency, sequencing of primer binding regions is key to ensure that current field strains are still detected by standard PRRSV diagnostics. Therefore, extensive sequence information given in this case report about the Austrian field isolate AUT15-33 is highly valuable.

An advantage of virus isolation is the availability of these isolates for further research, for molecular studies as well as for animal trials to evaluate vaccine efficacy. This is especially important since AUT15-33 has spread widely throughout Austria in the year following the outbreak described here, affecting not only PRRSV naïve but also vaccinated herds. The rapid spread of this strain in the field indicates advantages compared to other PRRSV-1 strains. Whether underlying mechanisms include, e.g., a faster replication, an easier entry into target cells, a more effective down regulation of innate immunity or stronger immune evasion properties remains to be elucidated in future experiments.

In this case report, we describe a PRRSV-1 outbreak in a piglet-producing farm that for Austrian standards was characterized by unusually severe reproductive losses and losses in nursery piglets. We link this information to the genetic and epidemiologic background of the virus strain and the in vitro characteristics of the virus isolate. In our hands this combination of clinical information with molecular biological analysis is key to modern PRRSV management.

**Abbreviations**

DON: Deoxynivalenol; ELISA: Enzyme-linked immunosorbent assay; ISH: In situ hybridization; LV: Lelystad virus; MLV: Modified live vaccines; ORF: Open reading frame; PAM: Porcine alveolar macrophages; PCV2: Porcine Circovirus type 2; PRRS: Porcine reproductive and respiratory syndrome; PRRSV: Porcine reproductive and respiratory virus; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; SV: Swine influenza virus; ZEA: Zearalenone

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Availability of data and materials
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Authors’ contributions
EK provided field samples and all clinical data. RB and HW analysed the samples histologically and provided histological pictures. AL was responsible for qRT-PCR. LIS and BL performed all other laboratory work and analysed the data. LIS, AL and TR interpreted the results and designed the figures. LIS and AL wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate
The present case report does not include experimental data and all laboratory analyses were performed as routine diagnostics during a clinical outbreak in the field. Therefore, animal ethics committee approval was waived. The owner of the case herd gave consent to publish of the data.

Competing interests
The authors declare that they have no competing interests.

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4. Discussion & Conclusion

Although PRRSV is of major importance to the pork industry in Austria and many other European countries, information on the geno- and phenotype of current strains is sparse. The aim of this thesis was to close this gap in knowledge.

Our first hypothesis was that PRRSV strains currently circulating in Austria differ substantially from Lelystad-virus-like strains and vaccine strains. We could confirm this hypothesis for all three PRRSV strains that were analysed (AUT13-883, AUT14-440 and AUT15-33). Though they belong to the same PRRSV-1 subtype, they differ markedly from the old strains. In phylogenetic analysis they cluster in different lineages and their identities to Lelystad virus are relatively low (between 87 and 88 %). Additionally, several more PRRSV strains that were detected in Austria in the last three years were analysed less detailed (not included in the manuscripts). None of them clustered in the same lineage with LV. In contrast, most of them clustered with one of the Austrian strains characterized in this thesis with identities between 92 and 99 %. We did find strains very close to vaccine strains (identities > 99 %) but only on PRRSV-vaccinating farms so a reversion to virulence is very unlikely. We conclude that current PRRSV field viruses in Austria are evolutionary far away from LV and vaccine strains.

An obvious assumption would be that recent field strains are too distantly related and hence not covered by commercial vaccines. Although this is a major concern, it is not necessarily true. Since knowledge on B or T cell epitopes is limited, no prediction of cross-protection can be made based on phylogenetic relationship or any other analysis (Murtaugh and Genzow, 2011). Nevertheless, our findings imply that PRRSV-1 strains circulating in the field are evolving away from the classical prototype and vaccine strains. It is likely to be only a matter of time until field strains develop mutations enabling them to evade immune responses triggered by vaccine strains. On the other hand, also other factors apart from genome sequence influence the outcome of vaccination. For the virulent strains AUT15-33 it has been observed that not only PRRSV unsuspicious farms but also vaccinated farms were struck by PRRS. The efficacy of vaccination depends not only on the vaccine strain but also on the vaccination scheme and regularity, on the overall animal health status and on management.
factors (especially biosecurity). In this context one has to discuss the metaphylactic vaccination approach that is commonly used in the field and was applied in the outbreak described in the second manuscript. Herds suffering from an acute PRRS outbreak are being vaccinated to prevent further spread of the field virus. This concept may be beneficial (Cano et al., 2007; Linhares et al., 2012) but a virological or immunological explanation is lacking. Not only is it questionable whether vaccine virus is able to replace field virus but also recombination has been reported in cell culture and in the animal (Li et al., 2009; Liu et al., 2011; Murtaugh et al., 2002). Beneficial effects on the immune response against PRRSV cannot be justified by neutralizing antibodies because they appear soonest four weeks post infection. Potentially other parts of the immune system, for example innate immunity, are triggered by the interventional vaccination and help to reduce virus shedding. In conclusion, the concept of vaccination against PRRSV infection is only partly understood. So far, despite the high evolutionary rate, evidence of field strains escaping vaccine-induced immunity is rare (Costers et al., 2010). Thus, protective immunity is probably directed against conserved regions of the viral genome. Since neutralizing antibodies only occasionally provide protection against heterologous strains (Pirzadeh and Dea, 1997; Vanhee et al., 2010; Vanhee et al., 2011), a major role of cell-mediated immunity is very likely. The new Austrian isolates described here allow the realisation of challenge experiments in order to evaluate the performance of commercial vaccines with regard to protection against current PRRSV-1 strains. This is especially important for strain AUT15-33, for whom vaccination failure has been reported from the field.

The second hypothesis stated that a repeated, independent introduction of novel PRRSV strains into Austria takes place, rather than an ongoing evolution based on few parental strains. The hypothesis was partly confirmed. All three strains that were analysed in detail clustered in new lineages that were not described for Austria so far. AUT13-883 and AUT14-440 are related to a German strain (GER09-613) and a South Korean strain (KNU-07) when performing phylogenetic analysis based on full genome sequences. AUT15-33 showed a rather distant relationship to Belgian strains when analysing ORF2-7 and a close relationship to Croatian strains when analysing ORF7, which was the only available part of the Croatian virus genomes. On the other hand, in further analysis of strains recently found in the field (not included in the manuscripts) similarities to the three isolates described in detail were striking. An evolution based on parental strains is therefore likely to happen. Most probably a combination of both routes occurs. From time to time totally new PRRSV strains are
introduced to Austria, for example from Croatia or other neighbouring countries. These novel strains then become a subject of evolutionary pressure when competing with the already existing viruses. They adapt by mutating and evolve away from their parental strains (92-97 % identity) but still cluster in the same lineage.

To confirm the origin of a specific PRRSV-1 isolate is very difficult. Compared to PRRSV-2, fewer sequence data are available while the degree of heterogeneity is higher. Phylogenetic analysis is complicated due to insufficient availability of sequences as seen in the case of the Croatian strains with only ORF7 sequences being available. Phylogenetic trees are mostly based on ORF5 or ORF7, which does not always lead to the same results (Stadejek et al., 2013). In this study also one Austrian field strain, AUT13-883, grouped differently when applying ORF5 instead of ORF7 or the full genome. Nevertheless, no evidence for recombination, the most likely cause, was found in AUT13-883. An explanation for this could be the low number of available PRRSV-1 full genome sequences, which compromises the significance of breakpoint analysis. Even if phylogenetic analysis based on ORF5 and ORF7 yields the same results, analysis based on full genomes might still be different. Whole genome sequencing was very tedious and expensive in the past and therefore seldomly performed. Nevertheless, it would be favourable to take 100 % of the genome in consideration, instead of 4 or 2.5 %. A new approach facilitating this is next generation sequencing (Kvisgaard et al., 2013). This technique also accounts for quasispecies. The fact that animals are infected with many similar virus strains at the same time is an important characteristic of PRRSV infection (Goldberg et al., 2003) but is usually not considered in phylogenetic analysis. Next generation sequencing gives the possibility to monitor not only a single PRRSV strain but the whole virus cloud. Other typing systems for PRRSV have been proposed (glycotypes, serotypes, RFLP) but could not prevail yet (Murtaugh et al., 2010). Phylogenetic analysis is very well suited for evaluation of genetic relatedness but does not correlate with virulence or cross-protection between strains. Therefore in this study the full genome sequence analysis was completed with studies on the in vitro and in vivo growth characteristics to display a broader picture of the strains and to provide well-characterized isolates for further research.

Thirdly, we hypothesized that novel PRRSV strains possess new beneficial properties that represent an evolutionary advantage. To verify this hypothesis, genomic sequences, phylogenetic analysis, growth in vitro and virulence were studied in detail. For the Austrian isolate AUT14-440 several deletions were found in comparison to Lelystad virus. A twelve
amino acids deletion within the overlapping region GP3/GP4 is the most pronounced and potentially most important one. In the same region deletions have already been described but only with a length of up to nine amino acids. This new property of AUT14-440 might be beneficial to the virus because the region was shown to be immunogenic and also a target for neutralizing antibodies (Meulenberg et al., 1997). It is therefore under selection pressure and likely to evolve to evade humoral immunity (antigenic drift). Furthermore the minor membrane proteins GP2-GP4 form a heterotrimer that is essential for virus entry into the cell by binding to CD163. Genomic mutations in this region, especially if they affect two proteins, might be able to broaden the range of target receptors. The other special property of AUT14-440, the spontaneous growth in MARC-145 cells, which is rather unusual for PRRSV-1 strains, hints towards this direction. For AUT15-33, so far no deletions have been found (in nsp2 and ORF2-7) but the virus has other special qualities. It was readily isolated on porcine alveolar macrophages and grows with a distinct cytopathic effect. Likewise, the phenotype, as described on the case farm and on other farms, is characterized by massive reproductive problems. In contrast to PRRSV-2 and PRRSV-1 subtype 2 and 3, PRRSV-1 subtype 1 strains were, especially in the last years, rarely reported to cause distinct clinical disease to the extent described for AUT15-33. Abortions and birth of weak piglets lead to losses of up to 90% in a single farrowing badge. Additionally, the virus strain spread quickly to other farms within the same region as well as nationwide, indicating advantageous properties compared to other field strains. Potential explanations are a more effective entry into cells, a faster replication or the ability to cope better with the host’s immune system. In conclusion, it was confirmed that novel PRRSV strains possess new properties. Whether this implies an evolutionary benefit is, until now, a matter of speculation and should be evaluated in further experiments.

Although the diversity of PRRSV with regard to genotype and phenotype is very high, no virulence factors could be determined so far. Not even the characteristic deletion in nsp2 of highly pathogenic PRRSV-2 strains could be proven to correlate with virulence in a reverse genetics approach. Considering the unsatisfying results of vaccination, improved knowledge on immunogenic or virulence-linked genome regions is urgently needed. Reverse genetics are a very suitable technique to evaluate the function of specific regions by modifying the genome with deletions, insertions or mutations. The characteristics found for strain AUT14-440 and AUT15-33 represent a basis for new hypotheses that can be tested with reverse genetics experiments. In preliminary studies, cDNA clones of isolates AUT14-440
and GER09-613 have already been constructed but are not functional yet. This may be due to incorrect genome sequences as a result of cloning of RT-PCR products that belong to different quasispecies and are not compatible with each other. In contrast to PRRSV-2, for PRRSV-1 few cDNA clones have been described in literature so far. Hence, there might be a general problem with replication efficiency or virus rescue of PRRSV-1 clones. Nevertheless, studies can be conducted with chimera viruses that consist of a functional PRRSV-2 cDNA clone (Truong et al., 2004) and the genome region of interest, for example the GP3/4 deletion of AUT14-440. First results indicate a good functionality of these chimeras. Another interesting question to address is the dominance that AUT15-33 showed when spreading in the field. Hopefully full genome analysis, potentially using next generation sequencing techniques, and animal experiments addressing the effects on the reproductive tract will give further insight into the molecular mechanisms. To facilitate future analysis of molecular functions of recent PRRSV-1 strains in future, improved serological tools are required. To this end several PRRSV-1 proteins were expressed and monoclonal antibodies against nsp4 and N protein were generated and characterized (not included in the manuscripts). The anti-nsp4 antibody is broadly reactive against both PRRSV species because it binds to a very conserved genome region. Against N protein, also a polyclonal serum reactive in immunofluorescence and western blot was produced. Since most research focusses on PRRSV-2 strains, serological reagents for PRRSV-1 are valuable tools to broaden the knowledge on PRRSV-1 in the future.

Taken together, this study provides well-characterized, current Austrian PRRSV field isolates. In addition to the strains described in the manuscripts (AUT13-883, AUT14-440 and AUT15-33), more Austrian field isolates were obtained and are available for further studies. A major advantage of isolating viruses is that genomic material is available in large amounts, which facilitates sequencing of larger genome fragments and especially next generation sequencing. Furthermore virus can be grown in cell culture and subsequently used in animal trials for infection or challenge experiments. Additionally to the evaluation of vaccines, the reliability of diagnostic methods can be tested and kept up to date with the new field isolates. They provide an insight into the actual PRRSV situation in Austria and represent the first published full genome sequences of Austrian PRRSV strains. Finally, the new field strains form an excellent basis for further research activities on molecular mechanisms of pathogenesis, virulence, immune evasion and persistence of PRRSV-1.
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6. Appendix

6.1 Supplementary material for manuscript in 3.1

6.1.1 Additional file 1
6.1.2 Additional file 2