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Virulence and Epidemiological Studies on the Novel Pestivirus Linda Virus

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Declaration

I hereby declare that the following PhD thesis has been composed in its entirety by myself and that the work presented herein is my own, except where stated otherwise by reference. The work has not been submitted, as a whole or in parts, for any other academic degree or professional qualification.
The rules of good scientific practice have been followed in all aspects at any time.

Alexandra Kiesler
Vienna, August 2022

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1. Summary / Zusammenfassung

1.1. Summary

Pestiviral infections rank among the most important viral diseases of the livestock industry worldwide. Classical swine fever virus (CSFV) poses a substantial threat to the pig production for more than 200 years and re-emergence in disease-free countries cannot be excluded.

With the advent of next-generation sequencing approaches, novel pestiviruses have been discovered in cloven-hoofed animals and other mammals. Of particular interest for the pig industry are novel atypical porcine pestiviruses, namely atypical porcine pestivirus (APPV) and Bungowannah virus (BungoV), that represent a potential risk for piglet producers. Another novel pestivirus was discovered in a single pig farm in the federal state Styria, Austria, in 2015 and termed “lateral-shaking inducing neurodegenerative agent virus” (LindaV) according to the clinical symptoms of congenital tremor (CT) in affected newborn piglets. Phylogenetic analyses revealed BungoV as the closest genetic relative of LindaV, with a nucleotide and amino acid sequence identity of approximately 70 %.

To elucidate the virulence of the novel pestivirus LindaV in immunocompetent pigs, a controlled acute infection experiment of post-weaning piglets was conducted. Infected animals did not show any clinical signs of disease, viremia was hardly detectable and virus replication was low. Seroconversion occurred between 7 to 14 days post infection and a strong neutralizing activity was detectable in all infected animals 21 days post infection. The anti-LindaV sera were further used to study the antigenic relationship between LindaV and other member species of the genus *Pestivirus* in cross-neutralization assays. Cross-neutralization was not observed and confirmed the far distant relationship.

Since its first description in 2015, LindaV was neither identified as an aetiological agent of CT cases in Austrian pig herds nor was there any evidence of its presence in other countries. Therefore, a retrospective seroepidemiological study was performed to gain insights into the prevalence of LindaV in the Austrian pig population between the years 2015 and 2020. Among a total number of 637 sera from sows and gilts, analyzed in a serum virus neutralization assay, a single positive serum was identified in a Styrian farm in 10 km distance to the index farm. These results confirmed the low seroprevalence of LindaV. Further analyses in this farm revealed a wider spread of LindaV in the pig herd in 2016 and a novel strain (LindaV strain Austria2) could be isolated.

In January 2021, a farm in Carinthia reported severe CT cases combined with a high preweaning mortality. A novel LindaV strain (LindaV strain Austria3) was identified as the aetiological agent of the disease outbreak and a diagnostic work-up revealed several highly viremic, seronegative six-week-old nursery pigs, shedding large amounts of virus in secretions and excretions. These findings stand clearly in contrast to the outcome of the acute infection experiment, and are a strong indicator for a chronic or persistent infection, as it is known for other pestiviruses.

In conclusion, we have demonstrated a low but steady prevalence of the novel pestivirus LindaV in the domestic pig population of Austria according to the results of the seroepidemiological study and the novel LindaV outbreak in 2021. As we could not identify a virus reservoir in domestic pigs, studies to elucidate a possible wildlife reservoir host need to be conducted in the future. The acute infection experiment showed that LindaV is a benign virus for immunocompetent pigs, but still induces high neutralizing antibody titers. Future transplacental infection experiments are needed to fulfil Koch's postulates.

1.2. Zusammenfassung

Pestivirale Infektionen zählen zu den wichtigsten Viruserkrankungen der Nutztierhaltung weltweit. Das Klassische Schweinepest-Virus (KSPV) stellt seit mehr als 200 Jahren eine erhebliche Bedrohung für die Schweineproduktion dar und ein Wiederauftreten in krankheitsfreien Ländern kann nicht ausgeschlossen werden.

Durch die breitere Anwendung der Next-Generation Sequencing (NGS) Technologie wurden neue pestivirale Sequenzen sowohl in Paarhufern als auch in anderen Säugetieren entdeckt. Von besonderer Bedeutung für die Schweineindustrie sind neuartige atypische porzine Pestiviren, namentlich das atypische porzine Pestivirus (APPV) und das Bungowannah-Virus (BungoV), da sie ein potenzielles Risiko für Ferkelproduktionsbetriebe darstellen. Ein weiteres neuartiges porzines Pestivirus wurde 2015 in einem Schweinebetrieb in der Steiermark (Österreich) entdeckt und entsprechend den klinischen Symptomen des kongenitalen Tremors (CT) bei betroffenen neugeborenen Ferkeln als „lateral-shaking inducing neurodegenerative agent virus" (LindaV) benannt. Phylogenetische Analysen zeigten, dass LindaV die höchste genetische Verwandtschaft zu BungoV aufweist, mit einer Nukleotidsequenz- und Aminosäuresequenzidentität von ungefähr 70 %.

Um die Virulenz von LindaV in immunkompetenten Schweinen zu bestimmen, wurde ein kontrolliertes akutes Infektionsexperiment in Aufzuchtferkeln durchgeführt. Infizierte Tiere

zeigten keine klinischen Symptome, Virämie war kaum nachweisbar und die Virusreplikation war sehr gering. Serokonversion erfolgte zwischen 7 und 14 Tagen nach der Infektion und ein hoher Titer neutralisierender Antikörper war bei allen infizierten Tieren ungefähr 21 Tage nach der Infektion nachweisbar. Die LindaV-Antiseren wurden darüber hinaus verwendet, um die antigenetische Verwandtschaft zwischen LindaV und anderen Pestiviren in Kreuzneutralisationstests zu untersuchen. Kreuzneutralisation konnte dabei nicht beobachtet werden, was die weit entfernte Verwandtschaft von LindaV zu den untersuchten Pestiviren bestätigte.

LindaV wurde seit seiner Erstbeschreibung im Jahr 2015 weder im Zusammenhang mit weiteren CT-Fällen in der österreichischen Hausschweinepopulation identifiziert, noch gab es Hinweise auf ein Vorkommen in anderen Ländern. Aus diesem Grund wurde eine retrospektive seroepidemiologische Studie durchgeführt, um Erkenntnisse über die Prävalenz von LindaV in der österreichischen Hausschweinepopulation zwischen den Jahren 2015 und 2020 zu gewinnen. Eine Gesamtzahl von 637 Seren von Sauen und Jungsauen wurde in einem Serumneutralisationstest analysiert. Dabei wurde ein einziges hoch neutralisierendes Serum, das aus einem steirischen Betrieb in 10 km Entfernung zur Erstbeschreibung stammte, identifiziert. Diese Ergebnisse bestätigen die bereits vermutete niedrige Seroprävalenz von LindaV. Weiterführende Untersuchungen zeigten eine weitere Verbreitung von LindaV in diesem Betrieb im Jahr 2016 und es konnte ein neuer Virusstamm (LindaV-Stamm Austria2) isoliert werden.

Im Januar 2021 meldete ein kombinierter Schweinebetrieb in Kärnten massive CT-Fälle verbunden mit einer hohen Saugferkelsterblichkeit. Ein neuer LindaV-Stamm (LindaV-Stamm Austria3) wurde als ursächlicher Krankheitserreger isoliert. Im Rahmen einer diagnostischen Aufarbeitung des Falles wurden mehrere hochgradig virämische, seronegative sechs Wochen alte Aufzuchtferkel identifiziert, die große Virusmengen im Speichel und Kot ausschieden. Diese Beobachtungen stehen im Gegensatz zu den Ergebnissen des akuten Infektionsexperiments und legen die Annahme nahe, dass es sich bei diesen Aufzuchtferkeln um chronisch oder persistent infizierte Tiere handelt, wie es von anderen Pestiviren bekannt ist.

Zusammenfassend lässt sich sagen, dass wir aufgrund der Ergebnisse der seroepidemiologischen Studie und des akuten LindaV-Ausbruches im Jahr 2021 von einer niedrigen, aber stetigen Prävalenz von LindaV in der österreichischen Hausschweinepopulation ausgehen können. Da in der Hausschweinepopulation kein Virusreservoir identifiziert werden konnte, sollten zukünftige Studien zur Aufklärung eines

möglichen Reservoirwirts in der Wildtierpopulation durchgeführt werden. Das akute Infektionsexperiment zeigte, dass LindaV ein schwach virulentes Virus für immunkompetente Schweine ist, aber dennoch hohe neutralisierende Antikörpertiter induziert. Zukünftige transplazentare Infektionsexperimente sind notwendig, um die Henle-Koch-Postulate vollständig zu erfüllen.

2. Introduction

2.1. The Genus Pestivirus

The genus *Pestivirus* forms together with the genera *Flavivirus*, *Hepacivirus* and *Pegivirus* the family *Flaviviridae* (Simmonds et al., 2017). Pestiviruses are a highly variable and fast growing group of enveloped, positive-sense, single-stranded RNA viruses with a diameter of 40–60 nm and typically infect animals of the order *Artiodactyla* (Blome et al., 2017a; Lindenbach et al., 2013). A unique feature of pestiviruses is the presence of the autoprotease N^{pro} and the envelope glycoprotein E^{rns}, which are absent in the other genera of the family *Flaviviridae* (Lindenbach et al., 2013). Currently, the genus *Pestivirus* consists of 19 approved species, that were recently classified as *Pestivirus A*-*S*, and several unclassified species (Postel et al., 2021). The classical and well-known pestiviruses include Bovine viral diarrhea virus 1 (BVDV-1, *Pestivirus A*), Bovine viral diarrhea virus 2 (BVDV-2, *Pestivirus B*), Classical swine fever virus (CSFV, *Pestivirus C*) and Border disease virus (BDV, *Pestivirus D*) (Smith et al., 2017). BVD and CSF rank among the most important viral diseases in the livestock industry and are listed diseases by the World Organization for Animal Health (OIE) (OIE, 2021). Disease outbreaks have a tremendous economic impact due to high mortality, culling of animals, reduced reproductive efficiency and trade restrictions of animals and animal products (Edwards et al., 2000; Houe, 2003; Richter et al., 2017).

Several so called atypical pestiviruses have been identified in domestic and wild even-toed ungulates. Pronghorn pestivirus (PAPeV, *Pestivirus E*) was only once isolated from a young, blind pronghorn antelope showing a high genetic divergence to the classical pestiviruses, but displaying some cross-neutralizing activity (Vilcek et al., 2005). Giraffe pestivirus (GPeV, *Pestivirus G*) was isolated from a giraffe suffering from a mucosal disease (MD)-like condition (Harasawa et al., 2000) and genetic and antigenic divergence to the classical pestiviruses was later shown (Avalos-Ramirez et al., 2001). Another distantly related atypical pestivirus, called HoBi-like pestivirus (HoBiPeV, *Pestivirus H*) was initially detected as a contaminant in fetal calf serum (FCS) from Brazil (Schirrmeier et al., 2004). Further reports of HoBiPeV genome in batches of FCS showed a worldwide distribution (Bauermann and Ridpath, 2015; Xia et al., 2011) and natural infections of cattle leading to clinical signs similar to BVDV infections were reported (Decaro et al., 2011; Weber et al., 2016). Aydin-like pestivirus (AydinPeV, *Pestivirus I*) was identified in sheep with symptoms and lesions reminiscent of BDV, but a closer genetic and antigenic relationship to CSFV than to BDV was subsequently shown (Becher et al., 2012; Oguzoglu et al., 2009; Postel et al., 2015).

The host range of pestiviruses expanded to other members of the class *Mammalia* with the discovery of rat pestivirus (RPeV, *Pestivirus J*) in *Rattus norvegicus* (Firth et al., 2014), and Rhinolophus affinis pestivirus 1 (BaPV, unclassified) in the bat species *Rhinolophus affinis* (Wu et al., 2012). Metagenomic sequencing approaches led to the identification of the complete (RPeV) and partial (BaPV) sequences of these two distantly related pestiviruses, but have not been associated with disease so far (Firth et al., 2014; Wu et al., 2012). The complete coding sequences of other pestiviruses in different rodents and bat species have been described (Wu et al., 2018b, 2018a), which were recently classified as *Pestivirus Q, R and S* (Postel et al., 2021). Most recently, a novel pestivirus was found in dead pangolins in China, termed Dongyang pangolin virus (DYPV, *Pestivirus P*) (Gao et al., 2020). Hemorrhagic and skin lesions were observed in affected animals and DYPV was detected in multiple organs, suggesting that the novel pestivirus may have been involved in the disease process (Gao et al., 2020). DYPV is far distantly related to the other pestivirus species and represents a distinct lineage (Gao et al., 2020).

In the last two decades several distantly related pestiviruses have been discovered in the host species swine. Bungowannah virus (BungoV, *Pestivirus F*) was identified in a pig farm complex in Australia in the year 2003 related to an increased number of stillborn and mummified piglets and sudden death in weaning age piglets (Kirkland et al., 2007). Diseased piglets showed a multifocal, nonsuppurative myocarditis and viral RNA was detected by *in situ* hybridization (ISH) (Finlaison et al., 2009). Hence, BungoV was determined as the causative agent of the "porcine myocarditis syndrome" (Finlaison et al., 2009). To date, there is no evidence that BungoV spread to other regions outside Australia (Abrahante et al., 2014; Cagatay et al., 2018; Michelitsch et al., 2019; Mósena et al., 2020). Another porcine pestivirus was discovered by metagenomic sequencing of porcine sera in the USA in 2015 and termed atypical porcine pestivirus (APPV, *Pestivirus K*) (Hause et al., 2015). After its first description, APPV has been detected worldwide with high prevalences in the domestic pig population (Beer et al., 2017; Kaufmann et al., 2019; Mósena et al., 2018; Pedersen et al., 2021; Postel et al., 2017a; Schwarz et al., 2017; Yuan et al., 2017). APPV has been linked to the clinical picture of CT type A-II (Arruda et al., 2016; De Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017). A diagnostic workup of CT cases associated with a high preweaning mortality in a piglet-producing farm in Styria, Austria in 2015 led to the identification of the "Lateral-shaking inducing neurodegenerative agent virus" (LindaV, *Pestivirus L*) (Lamp et al., 2017). The LindaV genome showed the highest nucleotide sequence identity to BungoV (68 %), but the clinical signs were similar to APPV infections and histopathological examinations confirmed CT type

A-II in affected piglets (Lamp et al., 2017). Similar to BungoV, LindaV has not been found again since its first description.

Interestingly, a novel pestivirus that was found in stranded harbor porpoises (*Phocoena phocoena*), Phocoena pestivirus (PhoPeV, *Pestivirus M*), is the closest relative of the BungoV/LindaV clade forming a monophyletic group (Jo et al., 2019). Furthermore, PhoPeV lacks the autoprotease N^{pro} (Jo et al., 2019).

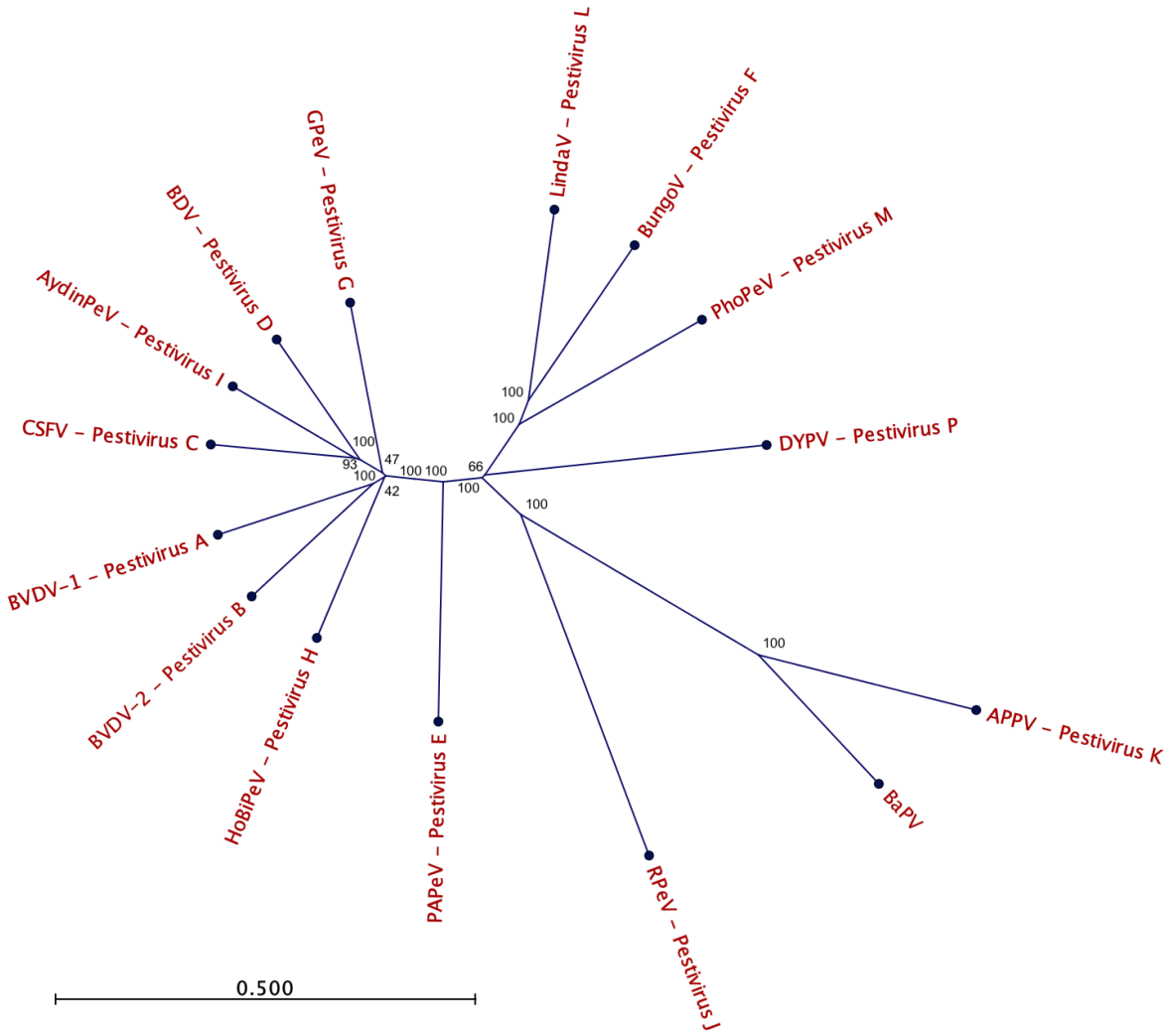

Fig. 1. Phylogenetic relationship between approved and unclassified pestivirus species. The unrooted, phylogenetic tree was constructed based on the full genome sequences using the neighbor joining algorithm and a bootstrap test with 1,000 replicates (CLC Sequence Viewer version 7.7.1, CLC bio/QIAGEN, Hilden, Germany). Bootstrap values are shown in percent and assigned to each node. The scale bar indicates the number of substitutions per site. The following GenBank accession numbers were used: BVDV-1 NADL (M31182, Bovine viral diarrhea virus 1, *Pestivirus A*), BVDV-2 890 (U18059, Bovine viral diarrhea virus 2, *Pestivirus B*), CSFV Alfort_187 (X87939, Classical swine fever virus, *Pestivirus C*), BDV X818 (AF037405, Border disease virus, *Pestivirus D*), PAPeV (NC_024018, pronghorn antelope pestivirus, *Pestivirus E*), BungoV (EF100713, Bungowannah virus, *Pestivirus F*), GPeV (NC_003678, giraffe pestivirus, *Pestivirus G*), HoBiPeV D32_00_HoBi (AB871953, HoBi-like pestivirus, *Pestivirus H*), AydinPeV (NC_018713, Aydin-like pestivirus, *Pestivirus I*), RPeV (KJ950914, rat pestivirus, *Pestivirus J*), APPV

AUT-2016_C (KX778724, atypical porcine pestivirus, *Pestivirus K*), LindaV (KY436034, Linda virus, *Pestivirus L*), BaPV (JQ814854, Rhinolophus affinis pestivirus 1, unclassified), PhoPeV (MK910229, Phocoena pestivirus, *Pestivirus M*) and DYPV (MK636874, Dongyang pangolin virus, *Pestivirus P*).

2.2. Genome Organization and Polyprotein Processing

The pestiviral positive-sense, single-stranded RNA genome consists of a single, large open reading frame (ORF) with an approximate length of 12 kb (Lindenbach et al., 2013). The ORF is flanked by 5'- (sequence length of 372–385 nt) and 3'- (sequence length of 185–273 nt) untranslated regions (UTR) (Lindenbach et al., 2013). The genome lacks a 5' cap structure and a poly(A) tail at the 3'-end (Brock et al., 1992; Lindenbach et al., 2013). An internal ribosomal entry site (IRES) is located in the 5' UTR, which forms a stem loop structure ("hairpin Ia") and mediates the translation process by binding the cellular 40S ribosomal subunit to the initial start codon AUG (López-Lastra et al., 2010; Poole et al., 1995; Rijnbrand et al., 1997; Yu et al., 2000). The ORF encodes a hypothetical polyprotein consisting of approximately 3900 amino acids, which is co- and posttranslationally cleaved into the eight nonstructural (NS) proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B and the four structural proteins core (C) protein, E^{rns}, E1 and E2 by cellular and viral proteases (Lindenbach et al., 2013).

The first viral protein N^{pro} (N-terminal protease) is an autoprotease and cleaves itself cotranslationally from the nascent polyprotein, generating also the N terminus of the C protein (Stark et al., 1993). The C protein, the envelope glycoproteins E^{rns}, E1, E2 and the nonstructural protein p7 are cleaved by cellular signal peptidases (SPs) from the polyprotein (Elbers et al., 1996; Harada et al., 2000; Rümenapf et al., 1993). Subsequently, the authentic C terminus of the C protein is generated by cellular signal peptide peptidases (SPPs) (Heimann et al., 2006), similar to the processing scheme of hepatitis C virus (HCV) (McLauchlan et al., 2002). The nonstructural proteins NS2-3, NS4A, NS4B, NS5A and NS5B, located in the C terminal part of the polyprotein, are cleaved by the NS3 serine protease (Tautz et al., 2000, 1997; Wiskerchen and Collett, 1991; Xu et al., 1997). NS4A acts as a cofactor at least for cleavage at the NS4B/NS5A and NS5A/NS5B sites (Tautz et al., 2000; Xu et al., 1997). Cleavage of the precursor protein NS2-3 is accomplished by the NS2 cysteine autoprotease (Lackner et al., 2004). The nonstructural protein NS3 is autocatalytically cleaved into a NS3 protease and helicase subunit (Lamp et al., 2013).

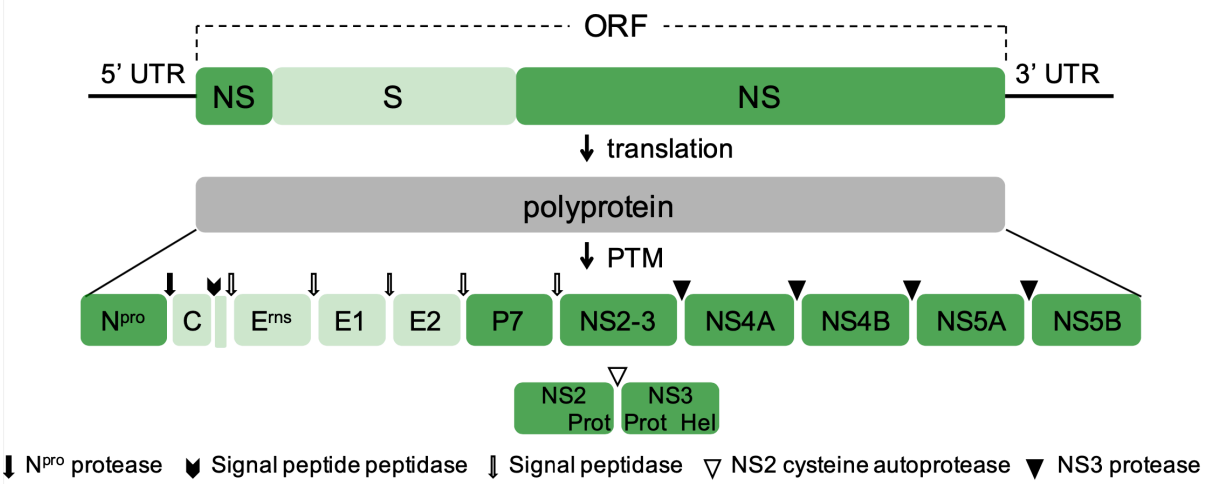

Fig. 2. Schematic representation of the pestiviral genome organization (top) and processing of the translated polyprotein (bottom). Arrows indicate cleavage sites of the pestiviral proteins. UTR, untranslated region; NS, nonstructural; S, structural; ORF, open reading frame; PTM, posttranslational modification; Prot, protease; Hel, helicase.

2.3. Pestiviral Life Cycle

The first step of the pestiviral life cycle is the attachment of the virion to the host cell surface. The attachment is mediated by the viral envelope glycoproteins E^{rns} and E2, which are located in the outer lipid envelope (Hulst and Moormann, 1997). The glycoprotein E2 not only plays a key role in viral attachment, but is also crucial for entry of the pestiviral virion into the host cell. Together with the viral envelope glycoprotein E1, E2 forms E1-E2 heterodimers linked via disulfide bonds, representing the functional fusion complex (Ronecker et al., 2008; Wang et al., 2004; Weiland et al., 1990). The E1-E2 fusion complex binds to specific host cell receptors. For BVDV, CD46 was identified as a host cell receptor (Maurer et al., 2004; Riedel et al., 2020), specifically the complement control protein (CCP) 1 module (Krey et al., 2006). Just recently, Cagatay et al. showed that CD46 acts as a cellular receptor for APPV, but not for CSFV and BungoV (Cagatay et al., 2021). Following binding of the pestiviral virion to the cell surface clathrin-mediated endocytosis is initiated (Grummer et al., 2004; Lecot et al., 2005). Due to the low pH environment in the endosome, fusion of the viral envelope with cellular membranes is accomplished and the uncoated nucleocapsid is released into the cytoplasm of the cell (Krey et al., 2005; Lecot et al., 2005). The positive-sense RNA genome serves directly as a messenger RNA (mRNA) for translation of the pestiviral proteins as described in section 2.2. The mature nonstructural proteins NS3-NS5B are necessary for replication of the viral RNA genome, of which the nonstructural protein NS5B serves as the RNA-dependent RNA polymerase (Murray et al., 2008). Following transcription of the positive-sense RNA genome

into a negative-sense RNA template strand, novel positive-sense RNA is generated by the viral replication machinery (Lindenbach et al., 2013).

Subsequently, the viral RNA is packaged into progeny viral particles in close association to membranes of the endoplasmic reticulum ("budding") and the mature infectious virions are released from the host cell via the secretory pathway (Lindenbach et al., 2013; Macovei et al., 2006; Schmeiser et al., 2014).

2.4. Pathogenesis and Immune Response

Following oronasal infection, the primary site of pestiviral replication are the epithelial cells of the tonsils (Blome et al., 2017b; Liess, 1987). Infection of lymphocytes in the surrounding lymphoid tissue disseminates the virus to regional lymph nodes, which is followed by a viremic phase and distribution of the virus to its secondary sites of replication, e.g. spleen or visceral lymph nodes (Blome et al., 2017b; Ganges et al., 2020; Liess, 1987). Besides epithelial cells and lymphocytes, pestiviruses infect a broad variety of cell types, e.g. cells of the mononuclear phagocytic system (i.e. macrophages and dendritic cells) or endothelial cells (Brodersen, 2014; Liess, 1987). It has been shown that cytokine secretion, such as TNF-α, IFN-α and different types of interleukin, plays an important role in the immune response against pestiviral infections (Bensaude et al., 2004; Chase, 2013). However, IFN-α overproduction has been linked to severe lymphopenia observed during the early phase of acute infections with highly virulent CSFV strains (Summerfield et al., 2006).

Animals acutely infected with CSFV or BVDV usually mount a long-lasting humoral immunity, which is detectable from day 10–14 post infection and is lasting for at least several years or even lifelong (Blome et al., 2017b; Fredriksen et al., 1999; Ganges et al., 2020; Lanyon et al., 2014a).

For APPV, immunological data are scarce and addressed mainly the humoral immune response. Cagatay et al. describe high levels of E^{rns}- and E2-antibodies after horizontal infection of pigs (Cagatay et al., 2019). Levels of E2-antibodies correlate with neutralizing antibody titers, indicating that the glycoprotein E2 is the major immunodominant antigen which is in line with findings of other pestiviral infections (Cagatay et al., 2019).

Maternally derived antibodies (MDA) provide an effective protection against infections at an early age. Nevertheless, high levels of circulating MDA can hamper the efficacy of vaccination procedures (Endsley et al., 2003; Vandeputte et al., 2001). MDA against BVDV have been detected in calves up to nine months of age (Al-Kubati et al., 2021), against CSFV up to at

least seven weeks of age (Vandeputte et al., 2001), and against APPV up to 21 to 48 days (Cagatay et al., 2019) or up to 8 weeks of age, respectively (Schwarz et al., 2017).

2.5. Persistent Infections

A unique feature of pestiviruses is their ability to cause persistent infections after intrauterine infection of the fetus during the critical stage of gestation before full maturation of the fetal immune system. Several studies addressed the time window of this critical stage of gestation, especially for the classical pestiviruses. For BVDV and BDV, approximately the first trimester of gestation was determined as the critical phase for the development of PI animals (up to day 125 of gestation for BVDV infections in cattle and up to day 60 for BDV infections in sheep) (McClurkin et al., 1984; Nettleton et al., 1992). Only non-cytopathic BVDV strains are able to cause persistent infections in the fetus (Brownlie et al., 1989). For CSFV, the critical stage was determined between day 50 and day 70 of gestation (Meyer et al., 1981; Moennig et al., 2003). A recent study about fetal infections with BungoV showed similar results to persistent infections with CSFV. Persistent infections in piglets occurred after fetal infection during days 35 to 55 of gestation (Finlaison and Kirkland, 2020a). Interestingly, several piglets presumably persistently infected with BungoV seroconverted after several months, indicating that these piglets were chronically infected and not PI animals by definition (Finlaison and Kirkland, 2020a).

2.6. Forms of Disease

Pestiviral infections can lead to a wide variety of clinical pictures depending on multiple factors, e.g. the route of infection (horizontal or vertical), the virulence of the virus strain, the age and the immunological and reproductive status of the infected individual.

In non-pregnant animals, acute BVDV infections mostly result in subclinical to mild disease (fever, depression, mild diarrhea and/or respiratory signs), but severe cases of pneumonia, enteritis and the fatal so-called “hemorrhagic syndrome” have also been described (Baker, 1995; Corapi et al., 1989; Lunardi et al., 2008; Yeşilbağ et al., 2014). Infection of pregnant cows may lead to infertility, embryonic or fetal death, mummification, malformation of the CNS or the skeletal system, or the birth of immunotolerant PI animals or clinically normal calves with pre-colostral antibodies, depending on the stage of gestation (Moennig and Liess, 1995). The lethal mucosal disease (MD) may arise in PI animals following mutation of the persistent non-cytopathic BVDV strain or superinfection with a cythopathic BVDV strain and is characterized by severe gastrointestinal lesions (Peterhans et al., 2010).

The outcome of CSFV infections highly depends on the virulence of the infecting virus strain and the age of the animal, among other factors (Blome et al., 2017b; Floegel-Niesmann et al., 2009, 2003). In acute infections, clinical signs vary from unspecific symptoms of the gastrointestinal or respiratory tract, fever and anorexia to cyanosis of the skin (especially of the acra), severe neurological disorders and peracute deaths (Blome et al., 2017b). Chronic infections mainly appear in pigs with a compromised immune system and are accompanied by unspecific disease signs (Blome et al., 2017b). Beyond that, chronically infected pigs show a poor growth and die after several months, in which they shed large amounts of virus (Blome et al., 2017b; Moennig et al., 2003). Infections of pregnant sows lead to similar disease signs as described for BVDV depending on the stage of gestation (Moennig et al., 2003). The so-called “late onset form of CSF” may develop in pigs persistently infected with CSFV and displays a similar situation to cattle persistently infected with BVDV regarding the virus reservoir in a population (Blome et al., 2017b; Moennig et al., 2003; Van Oirschot and Terpstra, 1977).

It was shown that BungoV leads to severe clinical disease after transplacental infection, resulting in an increased number of stillborn and mummified fetuses, and sudden death in piglets in combination with cardiorespiratory symptoms (Finlaison and Kirkland, 2020b). Persistent BungoV infections may develop after transplacental infection, resulting in stunted piglets shedding large amounts of virus over several months (Finlaison and Kirkland, 2020a, 2020b). Acute infection of immunocompetent pigs results in hardly any signs of disease, only mild diarrhea was observed (Finlaison et al., 2012).

Transplacental infection with APPV leads to CT type A-II in newborn piglets (Arruda et al., 2016; De Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017), and is occasionally seen concomitantly with the splay leg syndrome (Arruda et al., 2016; De Groof et al., 2016; Schwarz et al., 2017). Increased pre-weaning mortalities are usually related to starvation of the trembling piglets caused by the incapability of sucking milk. Experimental data about the outcome of acute APPV infections are very limited, but field observations indicate subclinical infections in adult pigs.

According to the first description of LindaV, the clinical picture is reminiscent of APPV infections (Lamp et al., 2017). Affected newborn piglets showed a severe lateral shaking of the whole body, which was histologically confirmed as CT type A-II (Lamp et al., 2017). In the field case, infected adult pigs did not show any clinical signs of disease. Experimental studies on the outcome of fetal or acute LindaV infections have not been conducted so far.

2.7. Diagnostic Methods

2.7.1. Direct Detection Methods

Direct detection methods are an important tool especially for the identification of PI animals, due to their long-lasting and high-level viremia and virus shedding. In live animals, whole blood, serum, plasma, peripheral blood mononuclear cells (PBMCs), secretions and excretions (saliva, nasal secretions, urine, feces, milk and semen) are suitable specimens (World Organisation for Animal Health, 2019a, 2019b). Additionally, in dead animals a broad variety of tissue samples (primarily lymphoid organs – tonsil, spleen, lymph nodes; kidney, lung, intestine and tissues of the central nervous system) can be used for direct detection (Lanyon et al., 2014a; World Organisation for Animal Health, 2019a, 2019b). Transiently infected animals are more difficult to detect, due to the short-term and low-level viremic phase (usually 7–10 days) and the low-level virus shedding (World Organisation for Animal Health, 2019a). Seroconversion in these animals 14–21 days post infection or a rise in the antibody titer using paired serum samples helps in identifying acutely infected animals (Lanyon et al., 2014a).

Currently, the detection of nucleic acids using quantitative reverse transcription PCR (RT-qPCR) assays is highly recommended for the diagnosis of BVDV and CSFV infections due to its high sensitivity and specificity and fast results (World Organisation for Animal Health, 2019a, 2019b). Detection of viral antigen by different antigen ELISAs (Ag ELISAs) is another rapid diagnostic method, but lacks sensitivity compared to the RT-qPCR (Blome et al., 2017b; Lanyon et al., 2014a; Postel et al., 2018; World Organisation for Animal Health, 2019a, 2019b). Virus isolation plays an important role in the confirmation of suspected cases and the propagation of novel virus isolates for further characterization, but takes days to weeks and usually requires a high level of infectious virus in the analyzed sample material (World Organisation for Animal Health, 2019a, 2019b). Other alternative direct detection methods are immunohistochemistry (IHC) and *in situ* hybridization (ISH) for BVDV diagnosis (Dubovi, 2013; World Organisation for Animal Health, 2019a), and the fluorescent antibody (FAT) or immunoperoxidase assays in cryostat sections of various tissues for CSFV diagnosis (Blome et al., 2017b; Postel et al., 2018; World Organisation for Animal Health, 2019b), but they are usually not used as sole diagnostic tools.

RT-PCR assays are used to amplify longer fragments of the genome, and amplicons are subsequently used for nucleotide sequencing and genetic and phylogenetic analyses. Phylogenetic analyses were usually performed based on short genomic fragments (e.g. 5' UTR, N^{pro}, E2 or NS5B coding region), whereas since recently longer parts of the viral genome

(e.g. N^{pro} – E2 coding region) or the complete viral genome are used (Becher et al., 1997; Fatima et al., 2021; Paton et al., 2000; Yeşilbağ et al., 2017).

2.7.2. Indirect Detection Methods

Animals acutely infected with pestiviruses typically develop a humoral immune response around 14 days post infection, which can be measured by a variety of serological tools (Lanyon et al., 2014a; Postel et al., 2018). Due to the lack of seroconversion in PI animals, these animals can only be detected in combination with direct detection methods (Lanyon et al., 2014a).

Different antibody enzyme-linked immunosorbent assays (Ab ELISAs) and serum virus neutralization (SVN) assays are most widely used for the detection of BVDV antibodies (Dubovi, 2013) and recommended by the OIE (World Organisation for Animal Health, 2019a). SVN assays show a higher specificity compared to the different Ab ELISA systems, but are more time-consuming and costly (Dubovi, 2013). Ab ELISAs enable a higher sample throughput and the use of milk and bulk milk samples as alternative specimens, which is especially important for herd level diagnosis (Lanyon et al., 2014b).

For the serological detection of CSFV infections, SVN assays and Ab ELISAs are also seen as the methods of choice (Postel et al., 2018; World Organisation for Animal Health, 2019b). Sera of pigs infected with other ruminant pestiviruses can lead to false-positive results and need to be confirmed in cross-neutralization assays (World Organisation for Animal Health, 2019b).

For research purposes, several Ab ELISA systems have been developed for the detection of antibodies against APPV based on the glycoprotein E^{rns} and E2 (Cagatay et al., 2019, 2018) and the nonstructural protein NS3 (Schwarz et al., 2017). The propagation of APPV in cell culture is difficult and inefficient, and serological methods based on the use of infectious virus are still a challenge (Arruda et al., 2016; Hause et al., 2015; Postel et al., 2016; Schwarz et al., 2017). To circumvent this problem, an indirect immunofluorescence assay based on a chimeric pestivirus (BVDV-1 backbone with the insertion of APPV glycoproteins E1 and E2) has been developed by Michelitsch et al. (Michelitsch et al., 2019). Nevertheless, Cagatay et al. established an APPV SVN assay using a high-passage, cell culture adapted APPV variant (Cagatay et al., 2019). For indirect detection of BungoV infections, a peroxidase-linked immunoassay (Finlaison et al., 2009) and an indirect immunofluorescence assay (Michelitsch et al., 2019) using an infectious BungoV isolate have been developed.

2.8. Epidemiology

In most European countries, North America and Australia CSFV has been eradicated in the last decades, but the virus is still endemic in the other parts of the world and reintroduction in disease-free countries is of great concern (Moennig and Becher, 2015; Postel et al., 2018). A few countries have successfully eradicated BVDV (e.g. Scandinavian countries and Austria) and control programs have been implemented in many countries, but BVDV is still highly prevalent in most parts of the world (Houe, 1995; Moennig and Becher, 2015; Moennig and Yarnall, 2021).

After the first description of APPV in the USA (Hause et al., 2015) the virus has been identified in domestic pigs worldwide (Beer et al., 2017; Dessureault et al., 2018; De Groof et al., 2016; Kaufmann et al., 2019; Mósena et al., 2018; Pedersen et al., 2021; Postel et al., 2017a; Schwarz et al., 2017; Yuan et al., 2021, 2017; Zhang et al., 2017) and in the wild boar population (Cagatay et al., 2018; Choe et al., 2020; Colom-Cadena et al., 2018; Stenberg et al., 2021). The prevalence of APPV in the pig population is high and seems quite variable, authors report genome detection rates between 2 % and 17 % (Postel et al., 2017a), between 9 % and 22 % (Beer et al., 2017), 19 % (Yuan et al., 2021), 13.9 % (Muñoz-González et al., 2017), 13 % in slaughter pigs and < 1 % in breeding pigs (Kaufmann et al., 2019). Additionally, seroprevalences of 16.3 % (Michelitsch et al., 2019) and ≥ 60 % were reported (Postel et al., 2017a).

Although several studies addressed the epidemiological situation of BungoV, it has never been detected anywhere else outside Australia so far (Abrahante et al., 2014; Kirkland et al., 2015; Michelitsch et al., 2019; Mósena et al., 2020). Epidemiological studies about LindaV are scarce and have only been conducted within the frame of investigations about the epidemiology of APPV (Cagatay et al., 2018; Kaufmann et al., 2019). Similar to BungoV, LindaV has not been detected outside the index farm.

2.9. Transmission and Control

Pestiviruses can be transmitted horizontally and vertically between individuals of a susceptible population. Horizontal transmission routes of BVDV and CSFV include direct contact of animals (e.g. nose-to-nose contact) and indirect transmission via contaminated feed, clothing, artificial insemination and airborne transmission (Kirkland et al., 1994; Laevens et al., 1999; Niskanen and Lindberg, 2003; Ribbens et al., 2004; De Smit et al., 1999). Vertical transmission to the fetus during gestation is a major transmission route of pestiviruses. The birth of PI

animals as “super-shedders” is the main driver for further in- and between-herd infections. Typically, the prevalence of PI animals is not higher than 1 % in an infected population (Houe, 1995). It is even more remarkable that this small proportion represents the main source for viral persistence in a population. Therefore, the identification and elimination of PI animals is a key element in successful eradication programs to interrupt the infection and transmission cycle (Moennig and Becher, 2018). Other important components of control programs include animal movement restrictions, high biosecurity measures and laboratory-based surveillance in the population (Moennig and Becher, 2018; Schweizer et al., 2021).

3. Aims and Hypotheses of the PhD Project

LindaV was discovered as a novel pestivirus in an Austrian pig farm in 2015 associated with CT type A-II in newborn piglets. Little was known about the virus, as it has never been described anywhere else before. A far distant genetic relationship to the other pestivirus member species based on the full-genomic sequences was shown and BungoV was identified as the closest relative of LindaV. The work of the PhD project was crucial to gather important information about the biological properties of LindaV, the distribution of the virus in the pig population and to get a clearer picture of the relationship between LindaV and other members of the genus *Pestivirus*.

The following aims and hypotheses of the PhD project were set to answer these questions:

- Aim 1: Determination of the virulence of LindaV and the humoral immune response against LindaV in experimentally infected immunocompetent pigs.
    a. We hypothesized that LindaV is a rather benign virus for immunocompetent pigs. However, acute infections presumably lead to seroconversion and high antibody titers should be detectable.
- Aim 2: Characterization of the antigenic relationship between LindaV and other pestivirus species in cross-neutralization assays.
    a. We assumed that LindaV shows a far distant antigenic relationship to other members of the genus *Pestivirus*, similar to the already demonstrated far distant genetic relationship.
- Aim 3: Determination of the prevalence of LindaV infections in the Austrian domestic pig population.
    a. We hypothesized that the prevalence of LindaV in the domestic pig population is very low, due to a lack of reports about disease outbreaks and first epidemiological results from other countries.

Furthermore, a novel disease outbreak of LindaV which occurred in 2020/2021 and its thorough diagnostic work-up provided more valuable insights into the features of this enigmatic novel pestivirus.

4. Manuscripts

4.1. Manuscript 1: Clinical and Serological Evaluation of Linda Virus Infections in Post-Weaning Piglets

viruses

MDPI

Article

Clinical and Serological Evaluation of LINDA Virus Infections in Post-Weaning Piglets

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Abstract: The novel pestivirus species known as lateral-shaking inducing neuro-degenerative agent (LINDA) virus emerged in 2015 in a piglet-producing farm in Austria. Affected piglets showed strong congenital tremor as a result of severe lesions in the central nervous system. Here, we report the results of a controlled animal infection experiment. Post-weaning piglets were infected with LINDA to determine the susceptibility of pigs, the clinical consequences of infection and the humoral immune response against LINDA. No clinically overt disease signs were observed in the piglets. Viremia was hardly detectable, but LINDA was present in the spleen and several lymphatic organs until the end of the experiment on day 28 post-infection. Oronasal virus shedding together with the infection of one sentinel animal provided additional evidence for the successful replication and spread of LINDA in the piglets. Starting on day 14 post-infection, all infected animals showed a strong humoral immune response with high titers of neutralizing antibodies against LINDA. No cross-neutralizing activity of these sera with other pestiviral species was observed. According to these data, following postnatal infection, LINDA is a rather benign virus that can be controlled by the pig's immune system. However, further studies are needed to investigate the effects of LINDA on the fetus after intrauterine infection.

Keywords: Linda virus; serological profile; virus neutralization assay; virus pathogenicity; humoral immune response

1. Introduction

The genus *Pestivirus* within the family Flaviviridae currently comprises 11 different species—recently termed *Pestivirus A–K* [1]. In addition to the long known classical swine fever virus (CSFV, *Pestivirus C*), a number of other pestivirus species have been identified in the porcine host in recent years, such as border disease virus (BDV, *Pestivirus D*), bovine viral diarrhea virus (BVDV-1, *Pestivirus A*), Bungowannah virus (BUNGO, *Pestivirus F*), and atypical porcine pestivirus (APPV, *Pestivirus K*) [1–5]. In 2015, we detected a yet unknown pestivirus species in a piglet-producing farm in Austria, which was termed lateral-shaking inducing neuro-degenerative agent (LINDA) virus [6]. Since the nucleotide sequence of LINDA shows a significant divergence of over 30% compared to the accepted pestivirus species, we proposed it as the new species *Pestivirus L* [1,6].

Pestiviruses are small enveloped viruses with a positive-sense, single-stranded, non-segmented RNA genome with a length of about 12 to 13 kilobases (kb) [7]. The genome consists of one large open reading frame (ORF), flanked by 5′- and 3′-non-coding regions [7]. This single ORF encodes a hypothetical polyprotein, that is co- and post-translationally processed into non-structural and structural proteins by viral and cellular proteases [8]. The three structural glycoproteins, termed E^{rns}, E1 and E2, and the nucleocapsid protein named Core are generated by cellular proteases [9,10]. The generation of the non-structural proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B is very complex. Multiple processing steps mediated by autoproteases (N^{pro} and NS2) and the major NS3/4A protease yield partially processed precursors, mature proteins and enzymatically active protein fragments [8,11–13]. The presence of the autoprotease N^{pro} and the envelope glycoprotein E^{rns} are recognized as characteristic of the genus *Pestivirus* [1,7]. Since the corresponding proteins have been found in the genome of LINDA, it can undoubtedly be classified in the genus *Pestivirus* [6].

CSFV is listed by the World Organization for Animal Health (OIE) as an economically important pig pathogen [14]. The clinical signs of classical swine fever (CSF) vary significantly depending on the virulence of the virus strain as well as the age and susceptibility of the infected pigs. CSF is usually characterized by fever, skin lesions, convulsions and, especially in young animals, death within a few days [15]. BUNGO emerged on a pig farm in Australia in 2003, causing an increased rate of stillbirths, mummification and sudden deaths of piglets [2,16]. Experimental studies were conducted to investigate the pathogenicity of BUNGO in weaner pigs and porcine fetuses under laboratory conditions. Despite the low pathogenicity of the virus in weaned piglets, a long-lasting viremia, efficient virus shedding and rapid seroconversion were detected [17]. In contrast, a multifocal non-suppurative myocarditis with myonecrosis was observed following direct fetal exposure to BUNGO mimicking intrauterine infection [18]. APPVs were discovered in the United States in 2015 by next-generation sequencing [4], and subsequently detected in many countries around the world [19–23]. A close correlation between intrauterine APPV infections and the occurrence of congenital tremor (CT) type A-II in newborn piglets was reported [24]. The simultaneous detection of nucleic acids of APPV and hypomyelination in the central nervous system of these piglets implied a causative role of APPV for the appearance of the so-called shaking piglet syndrome [20]. This causal relationship is further supported by the birth of shaking piglets after inoculation of pregnant sows with APPV-containing material [24].

LINDA was discovered during the investigation of an outbreak of CT in a piglet-producing farm. We identified the agent, isolated the virus, sequenced its genome and established a RT-PCR assay as well as serological reagents for its detection [6]. Since then, LINDA has not been found in any other farm in Austria or elsewhere in the world [25]. To gain a deeper insight into the biology of this virus, we infected weaned piglets with LINDA under controlled experimental conditions. The aim of this small-scale animal experiment was the determination of susceptibility, pathogenicity and virulence of LINDA in the immunocompetent porcine host. Sera from the experimentally infected piglets were further used to characterize the humoral immune response against LINDA and to study the induction of cross-neutralizing antibodies against other pestiviruses.

2. Materials and Methods

2.1. Cells and Viruses

SK-6 cells [26] and MDBK cells (ATCC® CCL-22™) [27] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FCS, Bio and Sell GmbH, Feucht, Germany; negatively tested for the presence of pestiviruses), 100 U/mL penicillin and 100 µg/mL streptomycin. All cells were maintained at 37 °C and 5% CO_2. Cell culture-derived LINDA was used for the experiments. After initial cell culture isolation from a clinical case (passage 1, P1), a primary LINDA stock was generated containing a 50% tissue culture infectious dose ($TCID_{50}$) of 1.1×10^7 (P2, GenBank® KY436034) [6]. The virus was titrated in an endpoint dilution assay and supernatant from a single focus was harvested (P3) to ensure freedom from other pathogens. A master stock (P4) was prepared and characterized by RT-PCR and subsequent Sanger sequencing. In direct comparison with the consensus sequence of the original isolate no mutations were detected. All LINDA infection doses used for animal inoculations were recovered from the master stock and thus represent cell culture passage 5 of LINDA. BVDV-1a strain NADL and BVDV-1b strain NCP7 were obtained from E. Dubovi (Cornell University College of Veterinary Medicine, Ithaca, NY) [28]. CSFV 2.3 Alfort-Tübingen [29], BDV-1 X818 [30], BVDV-2 strain 890 [31], and BVDV-3 (unpublished strain isolated from FCS, South American origin) were obtained from the virus collection of the Institute of Virology in Giessen (Justus-Liebig-University, Giessen, Germany). Pestivirus strain Giraffe-1 [32] was a gift from D. J. Paton, Animal Health and Veterinary Laboratory Agency (AHVLA, Weybridge, United Kingdom). BUNGO was obtained from stocks of the Elizabeth Macarthur Agricultural Institute (Department of Primary Industries, Menangle, New South Wales, Australia) [2].

2.2. Virus Infection and Titration

Infections of MDBK and SK-6 cells with various strains of the different pestivirus species were performed with the indicated multiplicity of infection (MOI). Virus stocks for the experiments were generated using 10-cm cell culture dishes infected with a MOI of 0.1. At 72 h post-infection, the cell culture supernatant was harvested, filtered through a 0.45 µm cellulose filter (Sartorius, Göttingen, Germany), aliquoted and stored at −80 °C until use. The $TCID_{50}$ of viral supernatants was determined in three replicates by an end-point dilution assay (EPDA). The virus titer was calculated using the Spearman-Kaerber algorithm [33]. Re-isolation of LINDA was performed using SK-6 cells and fresh sample material.

2.3. RT-PCR Detection

RNA was extracted from serum and tissue samples, saliva, feces, cultured cells or virus cell culture supernatant using the QIAamp Viral RNA Mini Kit and the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 60 µL RNase free distilled water and directly used for RT-PCR or stored at –80 °C for subsequent analysis. RT-PCR was carried out using the OneTaq One-Step RT-PCR Kit (NEB, Ipswich, USA) or the One Step RT-PCR Kit (Qiagen) using the oligonucleotides PPF 5'-GTKATHCAATACCCTGARGC-3' and PPR 5'-GGRTTCCAGGARTACATCA-3' [6]. PCR amplicons were subjected to gel electrophoresis and purified with the peqGOLD Gel Extraction Kit (Peqlab, Erlangen, Germany), if needed.

2.4. Calibration-Curve-Estimated Copy Number of LINDA Genome Equivalents

For the quantification of virus excretion, viremia and virus loads of organ homogenates, qRT-PCRs were performed on an ABI 7500 cycler (Applied Biosystems, Foster City, USA) using the LINDA and BUNGO specific primers LVqRTfor196 (5'-CACTGGWAAGGATCACCCACT-3') and LVqRTrev351 (5'-AATYACAACGGATAWTMTTTATACTGG-3') and the FAM/TAMRA labeled probe, LVqRTprobe322 (5'-Fam-ATAGGATGCCGGCGGATGCCCTGT-TamRa -3'). For the generation of a calibration curve, a T-vector plasmid fragment harboring the cDNA target sequence was produced using EcoRI digest, gel-purified and spectrophotometrically quantified. The copy number of recombinant plasmid fragment was calculated following the formula: N (molecules per µL) = (C

(DNA concentration in μg/μL) / K (fragment size in bp)) × 185.5 × 10^{13}. The factor results from the weight of the genome-equivalent ssDNA molecules (330 daltons per base), the volume projection factors and the Avogadro constant (6.02 × 10^{23} mol^{-1}). In order to obtain a standard curve, a ten-fold dilution series of the DNA control was included in the qRT-PCR setup [34]. Cycling conditions were 50 °C for 10 min, 95 °C for 1 min and 40 cycles of 95 °C for 10 sec, 60 °C for 1 min (amplification and fluorescence detection step). Semi-quantitative copy number estimation was calculated by 7500 System SDS Software (Applied Biosystems) based on the calibration curve. The amplification of the dsDNA standard does not include reverse transcription, which is why minor deviations may occur between RNA samples and dsDNA standard amplification depending on the efficiency of the cDNA synthesis. The qRT-PCR assay presented here is linear up to 10 copies of the dsDNA template per reaction (Ct value of 38). Since 10 copies per reaction were included as the lowest template amount in the standard series, this value represents the limit of quantification and our lower assay cutoff. The genome equivalents of 1.0 μL of purified RNA were converted to copies per 1 mL liquid sample, 1 g tissue sample or copies per swab using appropriate projection factors. The samples from the animal experiment were measured once due to high numbers and limited financial resources. However, the qRT-PCR data were validated by virus isolation experiments carried out in parallel with all samples obtained during the experiment.

2.5. Virus Neutralization Assay

All virus neutralization assays (VNA) were prepared in triplicate in 96-well microtiter plates. Viruses used in the VNA were diluted in DMEM without FCS from stock solutions generating a final titer of 100 to 300 $TCID_{50}$ per 0.1 mL. Initial two-fold dilution series of the serum samples were prepared with DMEM without FCS generating a final serum dilution of 1:256 in the last wells. Highly reactive sera were further diluted in five-fold series to a final serum dilution of $1:10^{5.6}$ in the last wells. 100 μL of each serum dilution were mixed with 100 μL of the respective virus solution containing between 100 to 300 $TCID_{50}$. After the virus was added, the VNA was incubated for 2 h at 37 °C in 5% CO_2. One hundred microliters of this virus/serum mixtures were added to 96-well flat bottom plates containing confluent cell monolayers and incubated for 48 h at 37 °C in 5% CO_2. Viral infections were detected by indirect immunofluorescence using murine monoclonal antibodies (MAbs) as indicated below. Back titration of each virus solution was performed in parallel. Defined positive and negative sera against the respective virus or groups were used as controls. The titers obtained from the VNAs were calculated using the Spearman-Kaerber algorithm and reported as the reciprocal of the serum dilution that inhibited infection of 50% of the cells (neutralization dose 50%, ND_{50}).

2.6. Indirect Immunofluorescence Assay and Antibodies

The immunofluorescence assays were performed as previously described [8]. Briefly, the cells were fixed with 4% paraformaldehyde for 20 min at 4 °C, permeabilized with 1% (vol/vol) Triton-X 100 (Merck, Darmstadt, Germany) in PBS and stained with the mouse MAb 6A5 [35] and A18 [36]. The monoclonal antibody 6A5 was used to detect the E2 molecule of BVDV-1, BVDV-2, BVDV-3, BDV, BUNGO, giraffe pestivirus and LINDA infections. CSFV E2 was detected by MAb A18. Goat anti-mouse IgG conjugated with Cy3 (Dianova, Hamburg, Germany) was used as a secondary antibody. A porcine BUNGO antiserum (748-09.10-1) collected from a naturally infected sow, which had produced an abnormal litter, was kindly provided by the Elizabeth Macarthur Agricultural Institute.

2.7. Animal Experiment

The animal experiment was approved by the ethics committee of the University of Veterinary Medicine, Vienna and the Federal Ministry of Science, Research and Economy according to the §§ 26ff. of the Austrian Animal Experiments Act from 2012 (Permission code: BMWF-68.205/0130-WF/V/3b/2017, permission date: 07.07.2017). Post-weaning crossbreed piglets (*Sus scrofa domestica*) were used for the study of LINDA pathogenesis and virulence in immunocompetent hosts. The pigs came from the breeding farm Medau of the University of Veterinary Medicine, Vienna. The entire herd of the

farm was negative tested for swine pathogenic influenza A viruses (IAV; H3N2, H1N1, H1N2 and H1N1pan) and porcine reproductive and respiratory syndrome viruses (PRRSV). The mother sows of the experimental animals were vaccinated against parvovirus and erysipelas during lactation according to the manufacturer's instructions (Parvoruvac; Merial GmbH, Hallbergmoos, Germany). The piglets themselves were protected on day 21 with a combined vaccination against *Mycoplasma hyopneumoniae* and porcine circovirus-2 (Circoflex and Mycoflex; both from Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany). No further special diagnostic tests were carried out as there were neither pathological nor clinical signs of disease in these piglets.

A week prior to the beginning of the trial (study days –7 to –1) 21 weaned piglets in the 13th week of life were housed in a biological safety unit (BSL-2) for adaptation. At the beginning of the animal experiment, six piglets were housed separately in order to later serve as sentinel animals. The remaining piglets were divided into three groups of five animals each and housed in separate units. One group was not infected and served as a negative control, one group was inoculated intramuscularly (i.m.) with 1×10^7 $TCID_{50}$ LINDA and the last group was infected intranasally (i.n.) with 1×10^7 $TCID_{50}$ LINDA (study day 0). One day after infection (study day 1), three sentinel animals were added to each of the infection-groups. A daily clinical score was determined for each individual animal. The general condition, behavior, body temperature, feed intake and weight gain of all animals were assessed and measured after the infection over a period of 28 days. Particular attention was paid to the occurrence of signs of disease. Each animal was assigned a daily clinical score, which included individual values for behavior, feed intake, dyspnea, ocular and nasal discharge, coughing and diarrhea in the range from 0 (physiological) to 3 (severe clinical symptoms) according to an established evaluation scheme of the University Clinic for Swine of the University of Veterinary Medicine, Vienna [37]. While the body temperature of all animals was also monitored daily, the body weight was assessed at the time point of arrival and on study days 0–7, 9, 14, 21 and 28. Blood and fecal samples as well as nasal and oral swabs were taken on the study days 0, 3, 5, 7, 14, 21 and 28/29. Urine samples (spontaneous urine samples or collected via cystocentesis) were obtained on study day 3 from most animals. All animals were euthanized with T61 (5.0 mg/mL tetracaine hydrochloride, 50 mg/mL mebezonium iodide and 200 mg/mL embutramide; 1 mL/10 kg) on study day 28 or 29 under general anesthesia (1.3 mg/kg azaperone and 10 mg/kg ketamine hydrochloride). During necropsy, organ samples were taken for molecular and pathohistological analysis. In particular, the LINDA RNA loads were analyzed in samples taken from the kidney, bladder, cerebellum, cerebrum, spinal cord, dorsal root ganglia, thymus, spleen, tonsils, lymph nodes (Lnn. inguinales, mesenteriales and mandibularis), parotid and sublingual glands, heart, lung, liver and intestinal segments from all animals.

2.8. Pathological Examinations

Tissue samples of the brain and spinal cord, dorsal root ganglia, liver, spleen, kidney, urinary bladder, thymus, spleen, tonsils, inguinal lymph node and mandibular gland were taken from all animals for histological examination. Additional samples of the coeliac ganglion, sciatic nerve, mesenteric and mandibular lymph nodes and parotid gland were included from the experimentally infected animals and sentinels. Five coronary sections of the brain were taken at the levels of telencephalon, diencephalon, cerebellum, mesencephalon and metencephalon. Transversal sections of the spinal cord were taken from cervical, thoracic and lumbar regions. The organ samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 2 µm and stained with hematoxylin and eosin (HE).

3. Results

3.1. Pathogenicity and Virulence of LINDA in Weaned Piglets

The animal experiment was performed with weaned piglets to investigate the clinical effects of LINDA infection in the immunocompetent host. The animals of all three groups (infected intranasally, intramuscularly or mock), including the sentinel animals, showed normal physiological parameters

and good general condition throughout the 28-day infection period. Neither the individually examined clinical values nor the additive clinical score showed major pathological changes in individual animals or significant changes between the different groups (Figure 1). Mild ocular and nasal discharge and cough were observed early after infection (study days 0 to 7) in most animals of both LINDA infected groups. However, these changes were also observed in sentinel animals of these groups that did not show seroconversion to LINDA (n.i. sent., described below). Mild fever (maximum rectal temperature of 40.7 °C) occurred in all i.m. infected animals within the first three days after inoculation. The single sentinel animal that later seroconverted showed an elevated body temperature and mild diarrhea. No differences were found between the groups in necropsy and histological examination. Gross examination revealed alveolar emphysema of the lung in animals of all the groups. Alveolar edema and pulmonary hyperemia were detected in some animals of all groups. Macroscopically, the central nervous system of all animals appeared normal. However, in the histological examination the majority of the animals from all groups (n = 17) showed mild, oligofocal, randomly distributed perivascular, mononuclear infiltrations and some glial nodules in the brain and/or spinal cord. A slight follicular hyperplasia of the spleen was observed in one sentinel animal of the i.n. and i.m. group. Stomach lesions such as ulcerations, hyperkeratinization or follicular hyperplasia were found in almost all animals. Mild, predominantly mononuclear, sometimes suppurating, interstitial nephritis or cortical infarction were found in many animals (n = 13) including animals from the non-infected control group. An interstitial lymphocytic infiltration of the mandibular gland was evident in one sentinel animal each of intranasally and intramuscularly infected group. An abscess was observed in the snout of an intranasally infected animal. The initial body weight of each animal was defined as 100% and the relative weight gain for each piglet and the average weight gain of the groups were calculated for each study day. The non-infected control group showed a slightly higher weight gain compared to the infected groups and the sentinel animals. However, the differences in weight gain between the experimental groups were neither pronounced nor statistically significant (Figure 2).

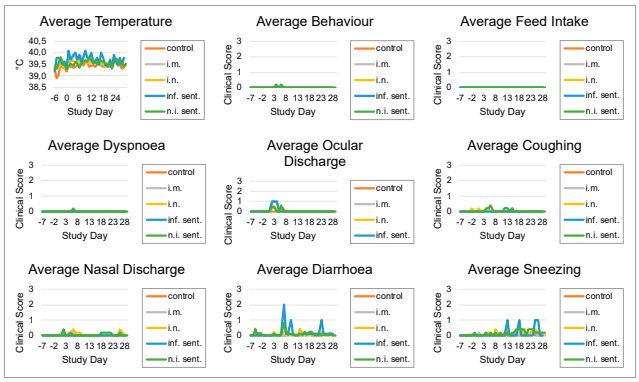

Figure 1. Pathogenicity of lateral-shaking inducing neuro-degenerative agent (LINDA) in immunocompetent piglets. A total of 21 piglets were divided in five groups: five negative control, five intramuscularly (i.m.) infected and five intranasally (i.n.) infected animals as well as three sentinel animals for the i.m. and three sentinel animals for the i.n. group. The animals from the i.m. and i.n. infection groups were inoculated with LINDA (1×10^7 $TCID_{50}$/mL) on study day 0. Body temperature, behavior, feed intake, dyspnea, ocular discharge, coughing, nasal discharge, diarrhea and sneezing were assessed daily and symptoms were classified by a scoring system with scores from 0 (physiological) to 3 (severe clinical symptoms). The mean of gathered parameters was calculated for the control, i.m. infected, i.n. infected, infected sentinel (inf. sent.) and non-infected sentinel (n.i. sent.) animals. No severe LINDA virus associated clinical signs were observed comparing infected and non-infected animals. However, mild fever and other signs of disease were seen in some infected animals, such as the infected sentinel animal within the i.m. group.

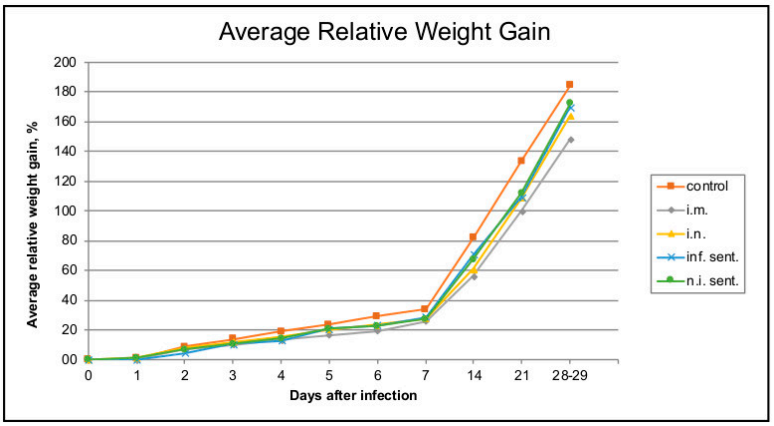

Figure 2. Average relative daily weight gain. Piglets were weighed on study days 0–7, 14, 21 and 28/29. The initial body weight was set as 100% value and relative weight gain was calculated for every individual at the indicated time-points. The mean value was calculated for every trial group. Infected groups show a slightly reduced weight gain compared to the negative control group after 7 days post-infection. However, the absolute differences between the groups were not significant.

3.2. Replication of LINDA in the Immunocompetent Porcine Host

Before the beginning of the experiment, blood samples of all animals were taken and tested for the presence of LINDA RNA and LINDA neutralizing antibodies. Serum samples were taken on study days 0, 3, 7, 14 and 28/29 of the experiment and analyzed in a LINDA-specific qRT-PCR assay as well as in virus isolation studies. No infectious LINDA or viral RNA were detected in the mock infected group at any time of the experiment. Most i.m. infected and all i.n. infected animals did not show a detectable viremia after LINDA inoculation. We observed a low level of viremia in two i.m. infected animals (5.0×10^5 GE/mL for animal 8 i.m. and 5.5×10^5 for animal 9 i.m. on study day 7) and a higher level in one sentinel animal of this group (2.3×10^7 GE/mL on study day 14, Figure 3). LINDA could be isolated from each of these qRT-PCR positive serum samples. Virus shedding was assessed using oral and nasal swabs as well as fecal and urine samples. Most of these samples gave negative results in the LINDA virus-specific qRT-PCR and virus isolation experiments. The oral swabs of one of the viremic piglets from the i.m. group (study day 7) and the oral and nasal swabs from three animals of the i.n. group gave signals below assay cutoff in the qRT-PCR assay (study days 3, 7 and/or 14). The RNA loads in these swabs were very low and cell culture virus isolation was not successful. An additional conventional RT-PCR was performed on the questionable samples as described before [6]. The amplification of LINDA-specific RT-PCR products was verified by nucleotide sequencing (Figure S1). LINDA RNA was not detectable in the fecal samples obtained on study days 3, 7 and 14 from the experimentally infected animals. However, the viremic sentinel animal (number 11) from the i.m. infected group showed substantial LINDA RNA loads in the feces (1.31×10^5 GE/g on study day 14) that also allowed successful virus isolation. All urine samples gave negative results in qRT-PCR and virus isolation. Multiple organs were sampled during necropsy on days 28/29 post-infection. Most organ samples gave negative results for the presence of LINDA RNA in the qRT-PCR. However, LINDA genomes were detectable in several lymphoid organs, such as the inguinal lymph nodes ($n = 4$), spleen ($n = 2$) and tonsils ($n = 4$) of animals of the i.m. infected group reaching values between 4.0×10^3 and 2.3×10^6 GE/g. LINDA RNA was also found in the infected sentinel animal of the i.m. group (animal 11) in the inguinal lymph nodes and spleen. Interestingly, the virus was detectable in the tonsils of all i.n. infected animals ($n = 5$), but only found in the inguinal lymph node of one of these animals ($n = 1$, Figure 3).

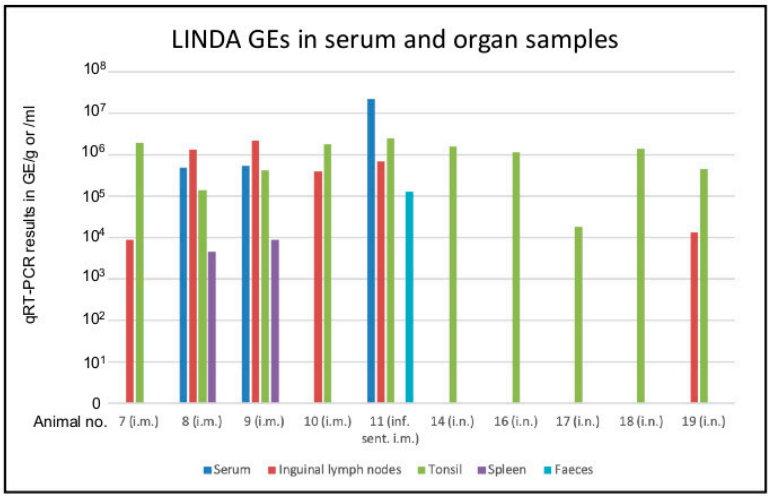

Figure 3. qRT-PCR results of the animal experiment. Multiple samples were analyzed for the presence of LINDA RNA before, during and after the experimental infection of piglets with LINDA. Blood and fecal samples as well as nasal and oral swabs were taken on study days 0, 3, 5, 7, 14, 21 and 28/29. Fecal samples were collected on study days 3, 7 and 14, while urine samples were only taken from most animals on day 3. Kidney, bladder, cerebellum, cerebrum, spinal cord, dorsal root ganglia, thymus, spleen, tonsils, different lymph nodes (Lnn. inguinales, mesenteriales and mandibularis), parotid and sublingual glands, heart, lung, liver and intestinal segments were sampled from all animals during necropsy on days 28/29. Most samples gave negative results for LINDA RNA. Only two i.m. infected animals showed detectable LINDA RNA loads in serum samples taken on study day 7 and one sentinel animal of the i.m. group on day 14. These serum samples also allowed the re-isolation of LINDA. Viral RNA was not detectable in urine samples and only detected in one fecal sample (animal 11, i.m. group, infected sentinel). However, the RNA of LINDA was detected in multiple samples from lymphoid organs demonstrating the presence of LINDA in the experimentally infected animals as well as in the infected sentinel animal until the end of the experiment.

3.3. Humoral Immune Response against LINDA

All serum samples obtained before, during and at the end of the experiment were tested for LINDA-specific antibodies using a LINDA virus neutralization assay (Figure 4). No virus neutralizing activity was measured in the serum samples taken before the start of the experiment (ND_{50} < 1/2, below limit of detection). Sera from the mock infected group and sera from the LINDA RNA negative sentinel animals showed no virus neutralizing activity at the end of the trial. A gradual onset of humoral immune responses was observed in all infected animals (i.m. and i.n.) between days 7 and 14 post-infection, reaching peak values of up to 1/8,640 ND_{50}/mL. A comparably strong reactivity of 1/1,028 was also seen in the serum sample of the i.m. infected animal 6, in which no LINDA RNA replication was detected throughout the experiment. Interestingly, the development of the humoral immune response was delayed to study day 21 in the LINDA infected sentinel animal 11.

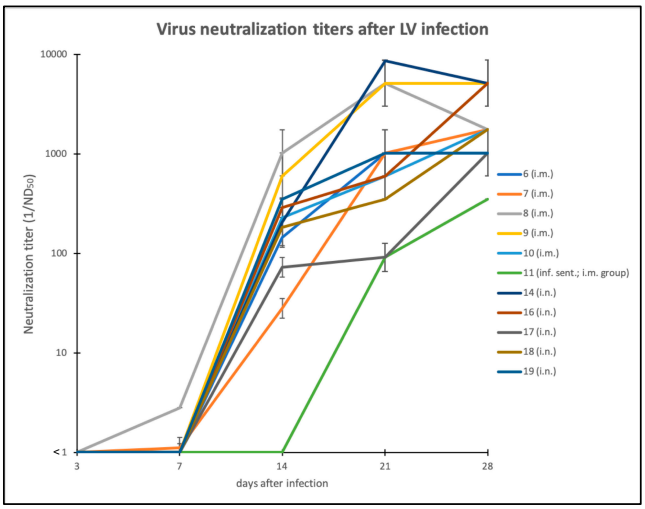

Figure 4. Virus neutralization activities of LINDA immune sera. The serum samples taken on study days 0, 3, 7, 14, 21 and 28/29 were tested in VNAs against LINDA. All VNAs were performed in triplicate and ND_{50} was calculated using the Spearman-Kaerber algorithm. The reciprocal ND_{50} value is presented for each serum sample including error bars for positive and negative standard deviation. All $1/ND_{50}$ values were less than 1 until study day 7, when the first weak neutralizing activities were measured in single animals. Significant neutralizing activities (ND_{50} > 1:10) were detected in all infected animals starting on day 14. Note the later onset of humoral immune response in the infected sentinel animal from the i.m. group on day 21.

3.4. Cross Neutralization of LINDA-Immune Sera with Other Pestivirus Species

To assess antigenic relations to other pestiviruses, we characterized the LINDA-immune sera from study days 28/29 for their cross-neutralizing activities against multiple pestiviral strains. In particular, we performed VNAs with the pestivirus species BVDV-1a (strain NADL), BVDV-1b (strain NCP7), BVDV-2 (strain 890), CSFV 2.3 (strain Alfort-Tübingen), BDV-1 (strain X818), pestivirus giraffe (strain giraffe 1), BUNGO and an unpublished BVDV-3 strain. We found no neutralizing activity of the LINDA-immune sera (ND_{50} < 1/2) against any of these viruses. Additionally, we tested a porcine BUNGO convalescence serum (748-09.10-1), initially obtained for immunodetection of BUNGO infections of cultured cells. This antiserum efficiently neutralized BUNGO (BUNGO ND_{50} 1/3200) as well as LINDA in our VNAs (LINDA ND_{50} 1/1,600) but had no effect on the infection of BVDV-1, BVDV-2 and CSFV (all ND_{50} < 1/2).

4. Discussion

The aim of this study was to investigate the pathogenicity of LINDA during post-natal infections and to characterize the humoral immune response against LINDA in order to obtain important basic data for sero-surveillance studies. Therefore, a small-scale animal experiment was set up using LINDA infections in post-weaning piglets. In this experiment, we found no evidence of severe acute disease in weaners caused by LINDA infection. Only mild clinical signs, such as mild fever, nasal discharge and mild changes in fecal consistency, were observed in single animals without significant influence on the growth rates of these piglets. Inflammatory infiltrates and glial nodules occurred in the brain and/or spinal cord of animals from all groups including the control group. Hence, these lesions were not associated with LINDA infection and were consequently interpreted as non-specific experimental background. Other findings of necropsy could be interpreted as random findings or might represent

stress-related diseases, such as the typical gastric lesions. However, productive LINDA infections were observed in all animals, which were experimentally inoculated with 1×10^7 $TCID_{50}$, regardless of the infection route used. LINDA virus excretion was detected using qRT-PCR in oronasal fluids of some infected animals and excretion via feces was documented for a single animal. Viremia was hardly detectable in the serum of most animals, while all except one experimentally infected animal (animal 6 i.m.) showed a long-lasting presence of the virus in the tonsils and/or lymphatic organs. Unfortunately, no peripheral mononuclear blood cells (PBMCs) were preserved from the experiment that could have been used for a potentially more sensitive virus detection in the blood [38]. Therefore, future studies, also including pregnant sows, will investigate whether LINDA virus is associated with PBMCs and whether the analysis of isolated PBMCs allows a more sensitive diagnosis. The infection of one sentinel animal in the i.m. group confirmed that infectious loads of virus were secreted in this simulated acute infection scenario. When we consider the positive detection of LINDA in serum, nasal secretions and feces of some infected animals, blood contacts or oronasal uptake of the pathogen may be considered as possible pathways of infection as also demonstrated by the successful artificial i.m. and i.n. infection. Since the minimum infection dose of LINDA for piglets is unknown, it is up to future studies to clarify the natural routes of infection. The clinical data are in accordance with clinical and epidemiological data about other pestiviruses, such as BVDV, BDV or CSFV, showing that mononuclear cells and lymphoid organs are the primary targets of pestivirus infections [39,40]. Most pestiviruses are well-adapted to their host species and the acute infection of immunocompetent animals usually leads to mild to subclinical disease with limited virus replication [41,42]. Severe pestiviral disease, such as abortion, malformation or neurological disorders, is mostly a consequence of intrauterine infections and responsible for the high economic losses following pestiviral infections [43]. This circumstance also explains the differences in the clinical picture between the animal experiment with immunocompetent piglets presented here and the LINDA virus outbreak in a piglet breeding farm from which LINDA virus was originally isolated [6]. Similar results were obtained in infection studies with BUNGO in weaner piglets, where few clinical signs, a short phase of viremia (3 to 10 days post-inoculation) and low levels of virus excretion were observed [17]. We conclude that LINDA is not only the closest genetic relative of Bungowannah virus but also shows similar pathogenicity in pigs. The results of our post-natal experimental infection demonstrate that the clinical picture of BUNGO and LINDA in immunocompetent animals is distinct from symptoms of piglets affected by CSFV strains of high and moderate virulence but might be similar to low virulence CSFV strains [44]. Future studies evaluating LINDA infections of the unborn fetus will be necessary to assess the potential hazard of LINDA [18,45].

The acute infection of piglets with LINDA led to the development of a strong humoral immunity starting at about seven days post-infection (Figure 4). Despite high titers of neutralizing antibodies, viral RNA persisted in the tonsils and lymphoid organs as has also been shown for other pestiviruses. This phenomenon has been described for several pestiviruses and studied in detail for BVDV pointing to a potential risk of virus transmission from convalescent animals [46]. We used the generated LINDA-antisera to evaluate antigenic cross-reactivity between LINDA and other pestiviruses. Unfortunately, we could not include APPV in these tests, because no APPV strain was available that showed the necessary infectivity in the cell culture [19]. Our VNA data clearly demonstrate that antibodies from acute LINDA infections do not provide protection against infections with classical pestiviruses. Therefore, a serological interference with established VNAs for CSFV diagnosis is unlikely. However, further studies are needed to evaluate possible false positive reactions in serological CSFV tests using a larger sample size of LINDA-antisera as well as highly reactive immune sera obtained from sows infected during pregnancy with viremic, persistently infected piglets. Our data support the hypothesis that LINDA forms an independent species (*Pestivirus L*) within the genus *Pestivirus* with a highly divergent antigenic profile. Interestingly, the high titer BUNGO-antiserum showed a strong cross-neutralization activity in VNA against LINDA, while low to absent neutralization profiles of BUNGO-antisera against other pestiviruses were observed in previous studies [47]. This result is puzzling, because it is in conflict with the results obtained with the LINDA-antisera. A possible

explanation could be that the species BUNGO and LINDA form an antigenic group within the genus *Pestivirus*, sharing conserved antigenic motifs important for virus neutralization. Again, future studies may answer this question by analyzing a larger sample size of BUNGO- and LINDA-antisera in cross-protection VNAs and, even more importantly, in controlled animal experiments.

5. Patents

The authors B.L., L.S. and T.R. are inventors on a patent on Linda pestivirus (PCT/EP2017/084453; Isolation of a novel pestivirus causing congenital tremor).

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/11/11/975/s1, Figure S1: Detection of LINDA virus by conventional RT-PCR in oro-nasal fluids.

Author Contributions: Conceptualization, K.S., L.S. and B.L.; Data curation, A.K. (Alexandra Kiesler), H.P., S.D, H.-W.C. and B.L.; Formal analysis, A.K. (Alexandra Kiesler), K.S., L.S., K.B. and B.L.; Funding acquisition, T.R. and B.L.; Investigation, A.K. (Alexandra Kiesler), K.S., L.S., K.B., H.P., E.S., S.D., S.H., A.K. (Andrea Klang), C.R., H.-W.C., M.M., P.K., H.W., A.L., T.R. and B.L.; Methodology, K.S., L.S., K.B., H.P., E.S., S.D., S.H., A.K. (Alexandra Kiesler), C.R., H.-W.C., M.M. and T.R.; Project administration, B.L.; Resources, P.K., H.W., A.L., T.R. and B.L.; Supervision, H.W., A.L. and B.L.; Validation, A.K. (Alexandra Kiesler), L.S., H.P. and B.L.; Visualization, A.K. (Alexandra Kiesler) and B.L.; Writing—original draft, A.K. (Alexandra Kiesler), T.R. and B.L.; Writing—review and editing, A.K. (Alexandra Kiesler), L.S., T.R. and B.L.

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4.2. Manuscript 2: Prevalence of Linda Virus Neutralizing Antibodies in the Austrian Pig Population

viruses

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Prevalence of Linda Virus Neutralizing Antibodies in the Austrian Pig Population

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Abstract: A novel pestivirus species, termed Lateral-shaking Inducing Neuro-Degenerative Agent virus (LindaV), was discovered in a piglet-producing farm in Austria in 2015 related to severe congenital tremor cases. Since the initial outbreak LindaV has not been found anywhere else. In this study, we determined the seroprevalence of LindaV infections in the domestic pig population of Austria. A fluorophore labeled infectious cDNA clone of LindaV (mCherry-LindaV) was generated and used in serum virus neutralization (SVN) assays for the detection of LindaV specific neutralizing antibodies in porcine serum samples. In total, 637 sera from sows and gilts from five federal states of Austria, collected between the years 2015 and 2020, were analyzed. We identified a single serum showing a high neutralizing antibody titer, that originated from a farm (Farm S2) in the proximity of the initially affected farm. The analysis of 57 additional sera from Farm S2 revealed a wider spread of LindaV in this pig herd. Furthermore, a second LindaV strain originating from this farm could be isolated in cell culture and was further characterized at the genetic level. Possible transmission routes and virus reservoir hosts of this emerging porcine virus need to be addressed in future studies.

Keywords: pestiviruses; Linda virus; seroprevalence; serum virus neutralization assay; novel Linda virus strain

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1. Introduction

Pestiviruses are enveloped, small viruses with a positive-sense, single-stranded RNA genome of about 12.3 to 13 kb length [1]. The RNA genome consists of one large open reading frame (ORF) that is flanked by a 5′- and 3′-untranslated region (UTR). The ORF codes for a polyprotein, which is co- and post-translationally processed into four structural proteins, namely Core, E^{rns}, E1 and E2, and eight non-structural proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [1]. Within the family *Flaviviridae*, the envelope glycoprotein E^{rns} and the auto-protease N^{pro} are a unique characteristic for the genus *Pestivirus*, although a recently discovered pestivirus species in toothed whales lacks the N^{pro} gene [2].

The genus *Pestivirus* currently comprises 11 different species—recently termed *Pestivirus A–K* [3]. In addition to the classical pestivirus species, bovine viral diarrhea virus 1 (BVDV-1, *Pestivirus A*), bovine viral diarrhea virus 2 (BVDV-2, *Pestivirus B*), classical swine fever virus (CSFV, *Pestivirus C*), and border disease virus (BDV, *Pestivirus D*), several

novel pestiviruses have been identified in different host species. Three new species of pestiviruses have been found in domestic pigs in the last two decades, causing various forms of disease. Diverse strains of atypical porcine pestiviruses (APPV, *Pestivirus K*) that induce congenital tremor of type A-II in newborn piglets after intrauterine infection have been identified in shaking piglets worldwide [4–6]. A multitude of studies on the prevalence of APPV revealed a wide geographic distribution and an overall high prevalence in the domestic pig population as well as in the wild boar population [7–11]. Currently, APPV is separated into three clades (Clade I-III) [12]. While in North America and Europe Clade I prevails, Clades II and III are abundant in China and neighboring countries. In contrast, the only known strain of the species Bungowannah virus (BungoV, *Pestivirus F*) appeared in Australia and caused the so-called porcine myocarditis syndrome [13]. BungoV became endemic in one of the affected farm complexes [14], but it has never been found anywhere else [8,10,15,16]. A further porcine pestivirus was identified in 2015 in Austria during a screening for APPV in samples of piglets with congenital tremor. The clinical symptoms of Linda virus are reminiscent of congenital tremor in newborn piglets, but the affected piglets showed a stronger shaking phenotype with a higher pre-weaning mortality rate and the identified pestivirus was therefore termed Lateral-Shaking Inducing Neuro-Degenerative Agent (Linda) virus (LindaV, tentatively *Pestivirus L*) [17]. Phylogenetically, BungoV and LindaV are more closely related to each other than to any other pestivirus. Interestingly, the newly discovered whale pestivirus Phocoena pestivirus belongs to the same branch as LindaV and BungoV [2]. As observed with BungoV, LindaV has never been detected again since its first description.

So far, epidemiological studies regarding the prevalence of LindaV in the pig population have focused on the detection of LindaV RNA in porcine serum samples [9,10]. Our recent results from animal studies demonstrated that acute infections with LindaV are difficult to detect in immunocompetent animals despite the persistence of the virus in the tonsils and lymphoid organs [18]. These findings are similar to acute BVDV infections, where direct virus detection by RT-PCR is a diagnostic challenge, as only very low viral loads are detectable in serum samples [19]. Therefore, the absence of LindaV RNA in serum samples is not likely to be sufficiently sensitive to conclude an absence of the virus in a population. An experimental infection of immunocompetent pigs with LindaV induced a strong humoral immune response with high neutralizing antibody titers, which presumably last for a longer period of time. Cross-neutralization of antibodies with other pestivirus species was not observed except for BungoV specific antibodies [18]. Therefore, epidemiological studies based on the detection of LindaV neutralizing antibodies represent a reliable tool to gain insights into the prevalence of LindaV infections in the pig population.

Serum virus neutralization (SVN) assays are seen as the gold standard in serological diagnostics of pestiviral infections with regard to specificity and sensitivity of antibody detection. The combination of different pestiviral species and strains in comparative SVN assays for the detection of potential cross-neutralization and high-titer specific neutralization allows a precise indirect virus diagnosis [20–22]. Unfortunately, SVN assays represent a laborious and time-consuming diagnostic test that limits the mass screening of serum samples. To overcome these limitations, a fluorophore encoding LindaV clone (mCherry-LindaV) was constructed based on an infectious cDNA clone of LindaV, which allows a direct readout of the assay without the need for immunofluorescence staining.

In this study, we assessed the seroprevalence of LindaV infections in the domestic pig population of Austria. Porcine serum samples from commercial pig farms were screened for the presence of LindaV specific neutralizing antibodies in an SVN assay using an mCherry-LindaV clone for rapid analysis. Additionally, the serum samples were analyzed in a LindaV specific RT-qPCR. The introduction of LindaV in naïve pig herds is a considerable threat, potentially leading to major piglet losses, as was seen on the originally affected farm. Therefore, knowledge of the presence of this virus in the pig population is of high importance for pig producers.

2. Materials and Methods

2.1. Cells

SK-6 cells [23] were grown in Dulbecco's modified Eagle's medium (DMEM, Biowest, Nuaillé, France) supplemented with 10% heat-inactivated fetal calf serum (FCS, Corning, Tewksbury, MA, USA; negatively tested for pestiviruses), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at 37 °C and a CO_2 concentration of 5%.

2.2. Indirect Immunofluorescence Assays

Indirect immunofluorescence assays were performed as previously described [17]. Briefly, the cells were fixed with 4% paraformaldehyde for 20 min at 4 °C, permeabilized with 1% (vol/vol) Triton-X 100 (Merck, Darmstadt, Germany) in PBS, and stained with the cross-reactive mouse monoclonal antibody (MAb) 6A5 (anti E2). Goat anti-mouse IgG conjugated with Cy3 (Dianova, Hamburg, Germany) or goat anti-mouse IgG conjugated with FITC (Dianova) were used as secondary antibodies. Cell nuclei were counterstained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 5 µg/mL for 5 min at room temperature.

2.3. Generation of a Full-Length Linda Virus cDNA Clone

The initial LindaV field isolate from a concentrated passage three master stock (5×10^8 $TCID_{50}$/mL) was chosen for our molecular cloning attempts to avoid cell culture adaptations within the genome of the virus. A total of 96 mL of a LindaV suspension (passage 4) was concentrated using ultracentrifugation applying an average centrifugal field of 95,800× *g* for 4 h at 4 °C (Beckman Type 45 Ti rotor, 35,000 rpm). The pellet was resuspended in 400 µL Hepes buffer (25 mM, pH 7.5) and the total RNA was purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). The LindaV genome sequence (GenBank accession number: KY436034.1) was presented earlier [17], allowing the design of oligonucleotides hybridizing with the 5′-end and the 3′-end of the genome. A full-genomic cDNA fragment was amplified by RT-PCR using oligonucleotides LindaV-5′-forw (5′-GTATAGCAGCAGTAGCTCAAGGCTG-3′) and LindaV-3′-rev (5′-GGGCCTCTTGGAACTGTAAGTAGTC-3′) and the One*Taq* One-Step RT-PCR Kit (NEB, Ipswich, MA, USA). A pBR322 derived vector already containing all the features necessary for RNA translation including an SP6 promoter and a XhoI site for linearization was amplified by extension PCR to provide homologous sequence patches for cloning of the cDNA. Q5 polymerase (NEB) was used for vector amplification together with the oligonucleotides LindaV-VGA-forw (5′-CAGTTCCAAGAGGCCCCTCGAGCTACCTC ACTAACG-3′) and LindaV-VGA-rev (5′-GCTACTGCTGCTATACTATAGTGTCACCTAAAT CGC-3′). The PCR products of 12.6 kb (viral cDNA) and 2.1 kb (vector) were purified (Monarch DNA gel extraction kit, NEB) and combined to generate plasmid pL588 using a DNA assembly reaction (NEBuilder, NEB). For differentiation of the cloned recombinant LindaV (recLindaV) and the LindaV field isolate, a novel MluI site at nt position 5587 was introduced. Extension PCR was performed with Q5 polymerase (NEB) and oligonucleotides LindaV-MluI-forw (5′-AAAACGCGTGGCGCTATGGTACACCTCAGAAAAACAGGTC-3′) and LindaV-MluI-rev (5′-TTTACGCGTGCCTGCTATGTTGACGGCTTCGGGATTTA TTAC-3′), which preserved the encoded amino acid sequence. The PCR product was digested with MluI, purified and ligated with T4 ligase (NEB) resulting in plasmid pL602. With the help of the primers LindaV-4868-forw (5′-CAGCAGACAGCAACAGTATAC-3′) and LindaV-5901-rev (5′-CTTCCCTGCCCCAGTTGCTAG-3′), a 1055 nt fragment was amplified flanking the MluI site. The RT-PCR products were diluted 1:10 in 1 x restriction enzyme buffer (NEB3.1, NEB), digested with MluI at 37 °C for 1 h and subjected to gel electrophoresis.

The viral cDNA clone was passaged in the *E. coli* strain HB101, DNA was prepared by standard methods and the genome of recLindaV was confirmed by sequencing.

2.4. RNA In Vitro Synthesis and Virus Rescue

Synthetic infectious RNA was produced as previously described [24]. Briefly, 2.5 µg DNA of the plasmids pL588 and pL602 were digested with XhoI and purified using phenol-chloroform extraction. The linearized plasmid DNA was transcribed into genomic recLindaV RNA using SP6 polymerase (NEB). A total volume of 50 µL of the transcription mixture was DNase digested. The RNA was purified with the RNeasy Mini Kit (QIAGEN), eluted in RNase free water, and diluted with water to a final concentration of 0.25 µg/µL. SK-6 cells were transfected with 2.5 µg of the synthetic RNA by electroporation as previously described [25] and incubated for 24 h until progeny virus was harvested from the supernatant.

2.5. Construction of a Fluorophore Labeled Full Genome Infectious cDNA Clone of Linda Virus

Based on the full genome infectious cDNA clone of LindaV (pL588, as described in Section 2.3), a fluorophore labeled infectious cDNA clone of LindaV was constructed by fusing the coding sequence of the fluorescent protein mCherry to the 5′-end of the LindaV E2 coding sequence behind the signal peptide (Figure 1). PCR products with overlapping ends harboring the mCherry sequence were generated with the primers mCherry-F (5′-TAATAGGGGGAGCCCAGGGTATGGTGAGCAAGGGCGAGGAG-3′) and mCherry-R (5′-GCAGTTCGAAGTTGCATTCAAGCTTGTACAGCTCGTCCAT-3′). The LindaV cDNA backbone was amplified with the primers LindaV-E2-F (5′-ATGGACGAGCTGTACAAGC TTGAATGCAACTTCGAACTGC-3′) and LindaV-E1-R (5′-CTCCTCGCCCTTGCTCACCAT ACCCTGGGCTCCCCCTATTA-3′). PCRs were performed using Q5 DNA Polymerase (NEB). The PCR fragments were assembled in a DNA assembly reaction (NEBuilder; NEB) according to the manufacturer's instructions.

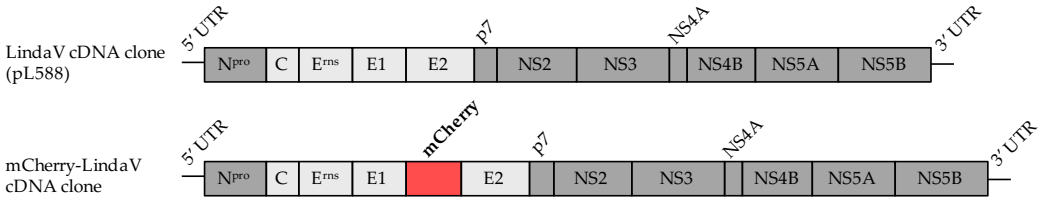

Figure 1. Schematic representation of the construction of an mCherry-labeled full genome infectious cDNA clone of Linda virus (LindaV). The coding sequence of the fluorescent protein mCherry was fused to the 5′-end of the LindaV E2 coding sequence in a LindaV cDNA backbone using a DNA assembly reaction. Non-structural protein coding sequences are shown in dark grey, and structural protein coding sequences in light grey. Lines represent 5′- and 3′-untranslated regions of the genome. UTR, untranslated region.

2.6. Serum Samples

The sample size for our sero-epidemiological study was determined based on the following Formula (1):

$$n \leq [1 - (1 - \varepsilon)^{1/d}] \times [N - (d - 1)/2] \quad (1)$$

where n = sample size number; ε = confidence level, set to 95%; d = number of diseased animals in the population; and N = population size [26]. Two different calculations with an assumed prevalence of 0.5% were made, one based on the total number of breeding animals in Austria (234,000 animals) and the other based on the total number of domestic pigs kept in Austria (2,770,000 animals). Both calculations resulted in an almost identical number of 597 and 598, respectively, porcine sera to be screened.

In total, 637 porcine serum samples from 132 pig farms, provided by the University Clinic for Swine of the University of Veterinary Medicine, Vienna and the Veterinary Health Service in Upper Austria, were screened for the presence of LindaV neutralizing antibodies

and LindaV RNA. The samples originated from sows and gilts from pig farms located in five federal states of Austria, namely Upper Austria (n = 335), Styria (n = 214), Lower Austria (n = 67), Carinthia (n = 11) and Burgenland (n = 10). The sera were taken by farm veterinarians during routine herd health monitoring visits between the years 2015 and 2020. Additional serum samples (n = 57) from the LindaV positive farm in Styria, identified during this study, were obtained and analyzed. Sera from the year 2016 (n = 30; 20 post-weaning piglets and 10 fattening pigs) were collected within the frame of a Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) circulation study, whereas sera from 2019 (n = 7; five post-weaning piglets, one gilt and one sow) and 2021 (n = 20, 10 fattening pigs and 10 of unknown origin) were sent in for diagnostic purposes. All serum samples were stored at −20 °C. Sera were heat inactivated for 30 min at 56 °C prior to conducting the SVN assays.

2.7. Serum Virus Neutralization (SVN) Assay

Initially, serum dilutions of 1/5 and 1/10 were prepared in DMEM without FCS in 96-well cell culture plates (STARLAB, Hamburg, Germany) in duplicate. An mCherry-LindaV stock (1.78×10^5 $TCID_{50}$/mL, determined by end-point dilution assay) was diluted to a titer of 100 $TCID_{50}$/50 µL. The test virus was added to the serum dilutions and incubated at 37 °C for 2 h. 1×10^4 SK-6 cells were seeded directly into the wells containing the pre-incubated serum/virus-mixture and grown for 72–96 h post infection. Defined positive and negative reference antisera, obtained from an experimental infection of immunocompetent pigs with LindaV [18], serum toxicity controls (serum dilution 1/5), cell controls and virus back titration controls were included in each SVN assay. Cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C, when a strong fluorescence signal was detectable in wells containing the negative reference sera and directly analyzed using a fluorescence microscope (Olympus IX70 fluorescence microscope; OLYMPUS, Hamburg, Germany).

Sera showing neutralizing activity in both initial dilutions were analyzed again in a five-fold serial dilution starting at a dilution of 1/5 and reaching a final dilution of 1/390,625. The 50% neutralization dose (ND_{50}/mL) was calculated using the Spearman-Kaerber method and expressed as the reciprocal (1/ND_{50}/mL) of the serum dilution.

2.8. RT-qPCR

Serum samples were pooled to a total volume of 140 µL (5 sera/pool; 28 µL per serum). Total RNA was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions. RT-qPCRs were performed on a Rotor-Gene Q cycler (QIAGEN) using the Luna Universal Probe One-Step RT-qPCR Kit (NEB). LindaV as well as BungoV specific primers and probe were used as previously described [18]. Sera of positive pools were extracted separately using 140 µL of each serum and again analyzed in RT-qPCR. The housekeeping gene beta-actin was used as an internal control for proof of successful RNA extraction and the absence of inhibitory factors in RT-qPCR. Amplification of beta-actin was conducted in a separate RT-qPCR run using the primers beta-actin-F1 (5′-CAGCACAATGAAGATCAAGATCATC-3′), beta-actin-R2 (5′-CGGACTCATCGTACTCCTGCTT-3′) and the probe beta-actin-HEX (5′-HEX-TCGCTGTCC ACCTTCCAGCAGATGT-BHQ-1-3′) under the same cycling conditions used in the LindaV RT-qPCR.

2.9. Two-Step RT-PCR and Sanger Sequencing

In order to obtain a full sequence of serum samples in which LindaV RNA could be detected, a set of primer pairs covering the full genome of LindaV was designed based on the available LindaV sequence in GenBank (accession number KY436034.1, oligonucleotides presented in Table S1). At first, cDNA was synthesized from 5 µL RNA using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. The cDNA was purified using Quantum Prep PCR Kleen

Spin Columns (Bio-Rad, Hercules, CA, USA) and 2.5 µL cDNA served as a template in subsequent PCRs using Q5 DNA Polymerase (NEB). PCR products were purified using the Monarch PCR DNA Cleanup Kit (NEB). Sanger sequencing of purified amplicons was performed by Eurofins Genomics, and sequence analysis was done using the DNA Strider 3.0 software [27,28].

2.10. Virus Isolation

A volume of 100 µL serum was used to inoculate 5×10^4 SK-6 cells, grown in DMEM with 10% FCS and penicillin/streptomycin on a 24-well cell culture plate. Cells were incubated at 37 °C and passaged every 72 h and cell culture supernatant was used to infect fresh cells in parallel. After every passaging, cells were examined for the presence of viral antigen in indirect immunofluorescence assays (as described in Section 2.2) using the cross-reactive mouse MAb 6A5 (anti E2) and goat anti-mouse IgG conjugated with Cy3 (Dianova) as a secondary antibody.

Additionally, total RNA was extracted from 140 µL cell culture supernatant using the QIAamp Viral RNA Mini Kit (QIAGEN) and successful virus propagation was identified by decreasing Ct values in the LindaV RT-qPCR.

2.11. Phylogenetic Analysis

Phylogenetic analysis of the novel LindaV strain (GenBank accession number: MZ027894) was performed using CLC Sequence Viewer 7.7.1 (CLC bio/QIAGEN Digital Insights, Aarhus, Denmark) based on the full-genomic nucleotide sequence or the polyprotein sequence. Sequences of approved and unclassified pestivirus species available in GenBank were used for sequence comparison. GenBank accession numbers of the respective pestivirus species are as follows: Linda virus (KY436034.1, tentatively *Pestivirus L*), Bungowannah virus (EF100713.2, *Pestivirus F*), CSFV Alfort_187 (X87939.1, *Pestivirus C*), BVDV-1 NADL (M31182.1, *Pestivirus A*), BVDV-2 890 (U18059.1, *Pestivirus B*), BDV X818 (AF037405.1, *Pestivirus D*), sheep pestivirus Aydin (NC_018713.1, *Pestivirus I*), pronghorn antelope pestivirus (NC_024018.2, *Pestivirus E*), reindeer pestivirus (AF144618.2, *Pestivirus D*), giraffe pestivirus (NC_003678.1, *Pestivirus G*), BVDV-3 D32_00_HoBi (AB871953.1, *Pestivirus H*), APPV AUT-2016_C (KX778724.1, *Pestivirus K*), *Rhinolophus Affinis* pestivirus 1 (JQ814854.1, unclassified), Norway rat pestivirus (KJ950914.1, *Pestivirus J*) and Phocoena pestivirus isolate NS170386 (MK910229.1, unclassified). Unrooted, neighbor-joining phylogenetic trees were constructed with bootstrap values based on 1000 replicates.

3. Results

3.1. Construction and Characterization of the Linda Virus cDNA Clone and the Fluorophore Labeled Linda Virus Clone

As a first step towards a fluorescent reporter virus, a full-genome infectious cDNA clone of LindaV was generated. LindaV RNA was purified and a full-length genomic PCR product was amplified by RT-PCR. The full-length genomic PCR product was purified and cloned into a minimalistic pBR322 vector backbone from the CSFV cDNA clone p447 (as described in [29]) in line with a SP6 promoter for in vitro RNA synthesis (pL588, recLindaV). A diagnostic MluI restriction enzyme recognition site was introduced in this cDNA copy of LindaV at position nt 5587 to differentiate between wild-type and recombinant viral RNA (pL602, recLindaV with MluI marker) (results are shown in Figure S1). The replication of recLindaV in SK-6 cells was demonstrated by an indirect immunofluorescence assay using MAb 6A5 (Figure S2). Growth curves of wild-type LindaV and recLindaV with or without the genetic marker were similar, with peak titers exceeding 1×10^7 $TCID_{50}$/mL measured at 48 h post infection (Figure 2).

Based on the LindaV cDNA clone pL588, a fluorophore labeled LindaV clone (mCherry-LindaV) was generated, where the mCherry coding sequence was inserted at the 5′-end of the LindaV E2 gene behind the signal peptide, according to recent publications on BVDV mCherry-E2 constructs [30,31]. Replication of the mCherry-LindaV in SK-6 cells

was observed in infected cells showing a strong cytoplasmic fluorescence signal at 48 h post infection (first fluorescence signals were visible approximately 12 h post infection) (Figure 3). Growth of the mCherry-LindaV in SK-6 cells was reduced compared to the parental recLindaV, with titers of 3.16×10^5 $TCID_{50}$/mL at 72 h post infection (Figure 2).

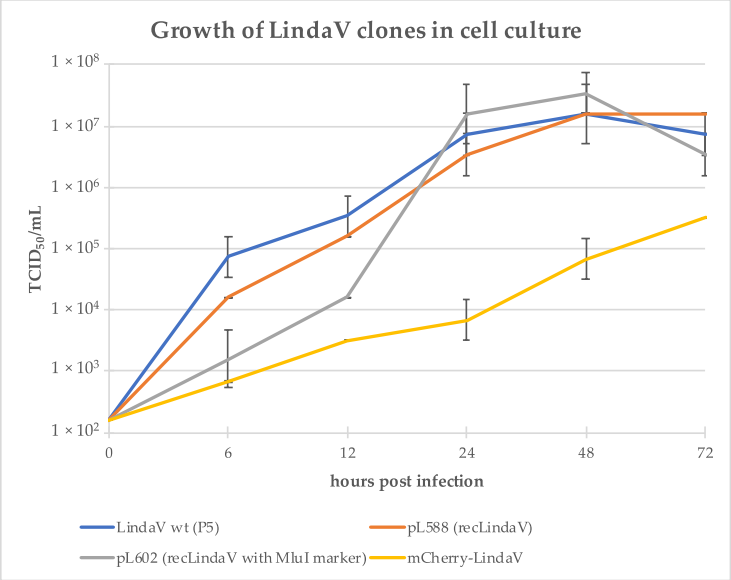

Figure 2. Cell culture growth of wild-type Linda virus (LindaV wt), recombinant Linda virus (recLindaV) and mCherry-Linda virus (mCherry-LindaV) clones. A monolayer of SK-6 cells was infected with 1×10^7 $TCID_{50}$ of the indicated viruses (MOI > 1). Two hours after infection, the cells were washed twice with DMEM without FCS and fresh cell culture medium was given. Cell culture supernatant samples were taken to analyze the progeny virus production after 0, 6, 12, 24, 48 and 72 h and titrated on SK-6 cells. Each titration was performed in triplicate and $TCID_{50}$/mL was calculated using the Spearman-Kaerber algorithm. No infectious virus was found at time-point 0 h, but the limit of detection was calculated with 1.58×10^2 $TCID_{50}$/mL. Error bars represent positive and negative standard deviations.

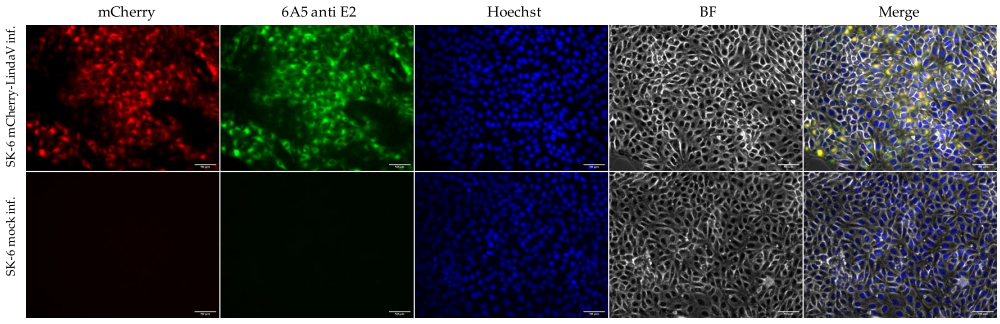

Figure 3. Indirect immunofluorescence assay of SK-6 cells infected with a fluorophore labeled Linda virus clone (mCherry-LindaV). SK-6 cells were infected with mCherry-LindaV and fixed at 48 h post infection. Cells were stained with the cross-reactive mouse MAb 6A5 (anti E2). Goat anti-mouse IgG conjugated with FITC was used as a secondary antibody. Cell nuclei were counterstained with Hoechst 33342. Images are shown at 20× magnification. Scale bars represent 50 µm. BF, brightfield.

3.2. Seroprevalence of Linda Virus in the Austrian Pig Population

The mCherry-LindaV clone was designed as a tool for the detection of LindaV specific neutralizing antibodies in porcine serum samples using an SVN assay. For validation, SVN assays with mCherry-LindaV and recLindaV were compared. Defined positive and negative reference antisera yielded the same results in both SVN assays (Figures S3 and S4), confirming the reliability of the SVN assay using mCherry-LindaV as a test virus.

Sample size calculations resulted in approximately 600 porcine sera that needed to be screened to determine the seroprevalence of LindaV infections in the Austrian pig population. A total of 637 serum samples from sows and gilts, collected between the years 2015 and 2020, was analyzed. The sera originated from 132 commercial pig farms located in five federal states of Austria. As the number of pigs in the three federal states Upper Austria, Lower Austria and Styria account for approximately 93% of the whole pig population in Austria, we aimed at screening a higher number of porcine sera from these areas, depending on the availability of archived sera from sows and gilts. Taking all these aspects into consideration, we analyzed 335 sera from Upper Austria, 214 sera from Styria, 67 sera from Lower Austria, 11 sera from Carinthia and 10 sera from Burgenland. One serum from 2019, originating from a sow housed in a pig farm in Styria (Farm S2), in the distant neighborhood of the initially identified LindaV positive farm (approximately 10 km distance between the two farms), showed a neutralizing activity in the screening assay. Further analysis in a five-fold serial dilution revealed a strong neutralizing activity of 1/2180 ND_{50}/mL (S2_S260, Figure 5). Two other sera from Farm S2 did not show any neutralizing activity (S2_S259 and S2_S261, Figure 5). Furthermore, no other serum of the 637 analyzed showed neutralizing activity against LindaV in the SVN assay (Figure 4).

For the detection of LindaV RNA, the 637 sera were pooled (5 sera/pool), total RNA was extracted and analyzed in a LindaV specific RT-qPCR. LindaV RNA could not be detected in any of the pooled samples. The housekeeping gene beta-actin was used as an internal control for the RT-qPCR analysis. All of the pooled sera yielded positive results in the beta-actin RT-qPCR (Ct values between 27 and 35). Three sera from Upper Austria could not be analyzed by RT-qPCR, because there was no material left for further analyses after performing the SVN assay.

The seroprevalence of LindaV was calculated to be 0.15% (1/637, based on the number of porcine sera screened) and 0.75% (1/132, based on the number of farms screened), respectively.

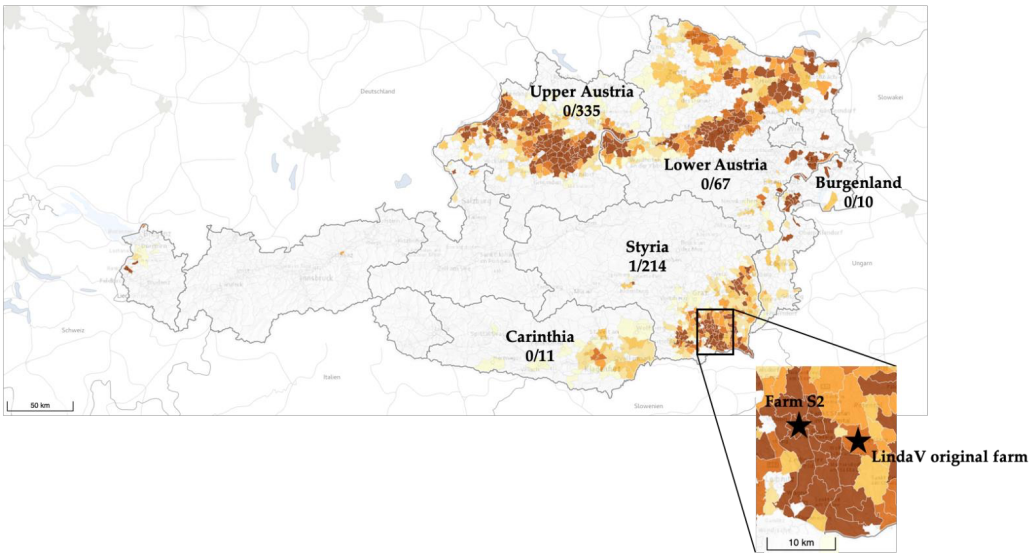

Figure 4. Presence of Linda virus (LindaV) neutralizing antibodies in porcine serum samples from Austrian pig farms. A total of 637 serum samples from five federal states of Austria (Upper Austria, Lower Austria, Styria, Carinthia and Burgenland) were analyzed in a LindaV SVN assay. The number of LindaV neutralizing antibody-positive sera and the total number of screened sera are given for each federal state. Colors indicate regions with high (dark brown color) and low (light brown color) pig density. The approximate locations of the antibody-positive farm (Farm S2) and the originally identified LindaV farm are marked with stars. (Modified from: https://www.statistik.at/atlas/?mapid=them_lw_as2010_viehbetriebe&layerid=layer1&sublayerid=sublayer0&languageid=0 (accessed 25 April 2021). © Statistics Austria—Cartography and GIS, created 1 September 2018).

3.3. Identification of Further Linda Virus Specific Antisera in Farm S2

Fortunately, Farm S2 has been monitored repeatedly in the past because of a PRRSV circulation project and several herd health screenings. This allowed an in-depth analysis of the LindaV prevalence on this farm. Archived sera from the year 2016 (n = 30, S2_S638-S2_S667), 2019 (n = 7, S2_S668-S2_S674) and recently sent in sera from 2021 (n = 20, S2_S675-S2_S694) were analyzed by SVN assays. In the sera of post-weaning piglets and fattening pigs from 2016, LindaV neutralizing activity could be detected. While an intermediate neutralizing activity was found in 20 sera (between 1/17.2 and 1/86.4 ND_{50}/mL), a high neutralizing activity between 1/968 and 1/2180 ND_{50}/mL was found in three sera and a low neutralizing activity of 1/1.538 ND_{50}/mL was detected in one serum, which can be considered as a negative result in the SVN assay. Three sera obtained in 2019, originating from a sow and two post-weaning piglets, showed an equally low neutralizing activity of 1/1.538 ND_{50}/mL, that were also evaluated as negative results. Neutralizing activity was not detectable in any of the sera obtained in 2021 (Figure 5).

All sera originating from Farm S2 were subjected to LindaV specific RT-qPCR. LindaV RNA could be detected in the serum from a post-weaning piglet from 2016 (S2_S641, Ct value 23), which did not show any neutralizing activity in the SVN assay. From the serum sample S2_S641 the full-genomic sequence of the virus was subsequently determined. A consensus sequence of 12,546 bp was established, missing only the ultimate ends of the 5′- and 3′-UTRs (GenBank accession number: MZ027894). Sequence alignment with the previously obtained LindaV sequence revealed a high identity of 98.54% based on the nucleotide sequences and of 98.37% based on the polyprotein sequences. Within the coding sequence of the envelope glycoprotein E2 we found a lower nucleotide identity of 97.87%

(E2 coding sequence) and of 95.47% (E2 amino acid sequence). A phylogenetic analysis with the approved and tentative pestivirus species clustered this novel LindaV strain (LindaV strain S2) with the already described LindaV prototype from 2015, branching with the species Bungowannah virus and the recently described species Phocoena pestivirus (Figure 6).

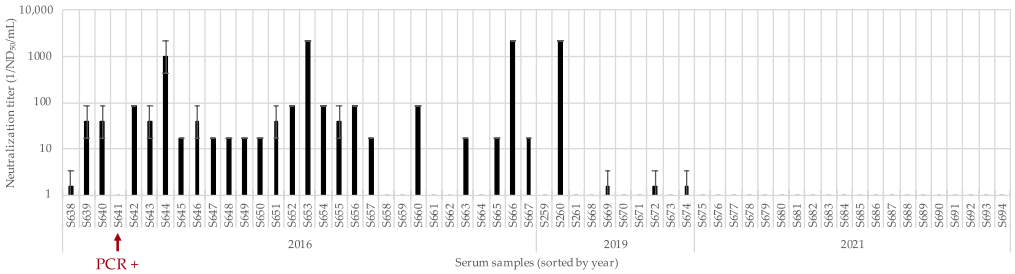

Figure 5. Virus neutralization titers of serum samples from Farm S2 from the years 2016, 2019 and 2021. Sera were analyzed in duplicate in a five-fold serial dilution starting at a dilution of 1/5 in an SVN assay. Neutralization titers (ND_{50}/mL) were calculated using the Spearman-Kaerber method. Neutralization titers are presented as the reciprocal ND_{50} value. Error bars indicate positive and negative standard deviations. Serum sample S641 is marked with a red arrow as yielding a positive result in the RT-qPCR assay.

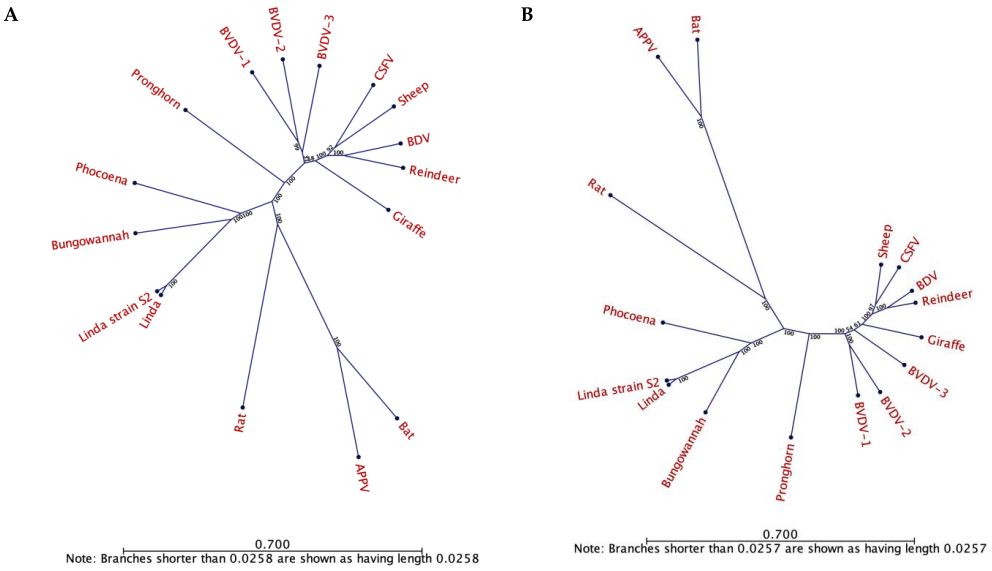

Figure 6. Phylogenetic analysis of the novel Linda virus strain S2 and approved and unclassified pestivirus species. Phylogenetic trees based on the full nucleotide sequence (**A**) and the polyprotein sequence (**B**) were constructed based on the neighbor-joining algorithm and bootstrap analysis with 1000 replicates and displayed as unrooted trees. Bootstrap values are indicated in percentage at each node. Scale bars indicate the number of substitutions per site. GenBank accession numbers are listed in Section 2.11. BVDV, bovine viral diarrhea virus; CSFV, classical swine fever virus; BDV, border disease virus; APPV, atypical porcine pestivirus.

Virus isolation by inoculation of SK-6 cells with serum sample S2_S641 was successful, despite storage at −20 °C for the last five years. Viral antigen in infected cells could be

detected in immunofluorescence assays using the cross-reactive anti-pestivirus MAb 6A5 (anti E2). Successful propagation in SK-6 cells could also be demonstrated with a decrease of the Ct value in RT-qPCR, starting with a Ct value of 23 in the serum sample and resulting in a Ct value of 19 after the third passage of LindaV-S2 in SK-6 cells.

4. Discussion

Classical swine fever or hog cholera has been known for 200 years and the causative agent was classified in the late 1980s as a member of the newly established genus *Pestivirus* within the family *Flaviviridae* [32]. Additional porcine pestiviruses were discovered in 2003 (Bungowannah virus, *Pestivirus F*) and in 2015 (Atypical porcine pestivirus, *Pestivirus K* and Linda virus, tentatively *Pestivirus L*) [6,13,17]. While APPV proved to be globally spread in wild and domestic pig populations and sporadically causes neurodegenerative disease, BungoV and LindaV were described only locally. With regard to LindaV, there is in fact no further report from abroad [9,10] and although we routinely screen for APPV and LindaV in our diagnostic laboratory, no further detection occurred. How can it be explained that BungoV and LindaV apparently do not spread among pig populations, despite their ability to efficiently infect and replicate in the porcine host [18,33] and the ability to establish persistence after fetal infection, as has been shown for infections of the porcine fetus with BungoV [34]? Data on the outcome of an experimental infection of pregnant sows with LindaV are still missing. However, high viral loads in the sera of diseased piglets in the initially affected farm indicate a persistent infection compared to the hardly detectable viremia in experimentally infected immunocompetent pigs [18]. As a first step to elucidate the spread of LindaV, we investigated the epidemiology of this novel pestivirus in the Austrian domestic pig population by assessing the seroprevalence of LindaV specific neutralizing antibodies. The results may appear fortunate, as we identified one highly neutralizing antiserum from archived samples of a single pig herd. The resulting seroprevalence of 0.15% can only be considered preliminary due to the small sample size and requires confirmation. On the level of examined herds the suggested prevalence is 0.75%.

These numbers are in contrast to the prevalence of APPV antibodies. Studies have demonstrated a wide distribution in several countries, ranging from 9–25% seropositive animals in Germany [8] and up to $\geq$60% in several countries of Europe, China and Taiwan [11]. While APPV antibodies were determined by antigen detection (immunofluorescence assays or ELISAs), LindaV specific antibodies were assessed by an SVN assay that provides the maximum specificity. In a previous report we have shown that no cross neutralization with other pestivirus induced antibodies exists for LindaV, except for a BungoV antiserum [18]. Prerequisite for SVN assays is an infectious system consisting of susceptible cells and infectious virus. LindaV can be easily propagated on porcine kidney cells (SK-6) without adaptation and reaches moderate to high titers [17]. For APPV, productive infectious systems have been put forward in the last years [35], so that SVN assays can be used to confirm earlier results.

For the SVN assay we designed a reporter system using a fluorophore labeled LindaV cDNA clone. The advantage is the easy readout of the fluorescent signal of infected cells without the need of laborious indirect immunofluorescence staining procedures. Construction of the reporter virus was achieved by the fusion of an mCherry gene to the 5′-end of the LindaV E2 gene directly downstream of the E2 signal peptide coding region analogous to previously published BVDV/mCherry-E2 constructs [30,31]. The resulting mCherry-LindaV clone displayed a retardation in virus multiplication, possibly due to the size of the introduced foreign gene, yet the 10-fold lower virus titers were sufficient for the establishment of a reporter SVN assay. It is currently undetermined which antibody specifications account for the neutralizing effect, but in analogy to other pestiviruses it can be expected that E2 represents the immunodominant antigen. Neutralization assays with E2 and E^{rns} affinity purified antibodies are planned.

The screening of serum samples from Austrian pig farms revealed a second LindaV affected farm in the distant neighborhood of the initial outbreak. After the detection of a seropositive sow in our initial screening, we could identify 27 (out of 57) additional positive sera from the years 2016, 2019 and 2021 with variable neutralizing antibody titers. LindaV RNA was detectable in one serum from 2016 and virus isolation from this serum was successful. From the data available, an outbreak of LindaV in this farm is likely to have occurred in 2016, because of a large number of sera showing intermediate to high neutralizing activity at this time point and the occurrence of an antibody negative and viremic post-weaning piglet. Most of the sera from 2019 showed no neutralizing activity and none of the sera from 2021 showed any neutralizing activity. This trend could indicate that a circulation of LindaV was present in the herd for at least three years, but that the virus disappeared from the farm in later years. It is not clear, whether the viremic post-weaning piglet represents a persistently infected animal or if it was in the viremic phase of an acute infection, as there were no follow-up samples available. According to the responsible herd veterinarian and the farmer, clinical signs of congenital tremor or increased pre-weaning mortality have never been observed in this pig herd. This would suggest an infection episode with LindaV during a time where no sows were in a critical stage of gestation, a subclinical infection or a possibly less virulent LindaV strain compared to the original strain. Nevertheless, the number of samples is not representative and we only have limited information about the situation on the farm farther in the past. Surveillance of the farm assessing the serostatus and possible presence of LindaV in the pig herd is underway.

While we have not identified a direct connection between the two farms, the relatively close proximity (approximately 10 km) suggests a local transmission of LindaV. Direct transmission via transport of live, infected animals or indirect transmission, as has been shown for other pestiviruses, like BVDV, CSFV or BungoV (reviewed in [14,36,37]), can be safely assumed. LindaV excretion in nasal secretions, saliva and feces has been demonstrated as a potential route for direct or indirect horizontal transmission [18]. Another possible route of virus transmission could be the transport of slaughter pigs to the slaughterhouse, as the loading of pigs from several farms together in one trailer is a common procedure in Austria due to the small farm sizes. However, the involvement of an unknown vector or an unidentified wild reservoir host cannot be excluded. In vitro studies have demonstrated a broad cell tropism of BungoV, which is in clear contrast to other pestivirus species [38]. Cell lines of human, monkey, mouse and bat origin were susceptible, raising the question of possible reservoir hosts and the origin of this virus [38]. We are currently looking into this with LindaV, but preliminary evidence suggests a narrower host species range than BungoV, at least on the level of susceptible cell lines.

Isolation and sequencing of the new LindaV strain revealed an identity of approximately 98% to the original LindaV (KY436034.1) based on the full genomic sequence. This high sequence identity combined with nucleotide exchanges that are regularly distributed along the whole genome, could indicate a low immune selection pressure on the virus. Nevertheless, we found a slightly lower sequence identity of 97.87% within the E2 coding region and of 95.47% within the E2 amino acid sequence. These results are not surprising, as the pestiviral glycoprotein E2 is the main target for neutralizing antibodies and therefore shows the highest variability within the pestiviral genome due to immune evasion strategies.

5. Conclusions

With a seroprevalence of 0.15% based on the animal level, our study confirms that LindaV is a rare pathogen in Austrian domestic pigs. In future experiments we will look at the prevalence of LindaV in boars as well as in the wild boar population using SVN assays. Further epidemiological and virological studies are required to decide whether the emerging pathogen LindaV remains a rare infection or has the potential for future epidemics.

6. Patents

The authors B.L., L.S., and T.R. are inventors of a patent on LindaV pestivirus (PCT/EP2017/084453; Isolation of a novel pestivirus causing congenital tremor).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13061001/s1, Figure S1: Establishment of reverse genetics for Linda virus (LindaV), Figure S2: Monoclonal antibody (MAb) 6A5 detects the E2 expression in cells transfected with recombinant Linda virus (recLindaV) in an indirect immunofluorescence assay, Figure S3: Serum virus neutralization assay (SVN assay) using an mCherry-Linda virus (mCherry-LindaV) and defined positive and negative reference Linda virus antisera, Figure S4: Serum virus neutralization (SVN) assay using a recombinant Linda virus (recLindaV) and defined positive and negative reference Linda virus antisera, Table S1: Oligonucleotides used in this study.

Author Contributions: Conceptualization, B.L. and T.R.; methodology, K.S., C.R., A.K. and B.L.; validation, B.L., T.R. and A.K.; formal analysis, B.L., L.S., K.S., A.K. and A.L.; investigation, B.L., L.S., K.S., A.K., M.M., J.P., T.R. and C.R.; resources, B.L., T.R. and A.L.; data curation, B.L., L.S. and A.K.; writing—original draft preparation, A.K., T.R. and B.L.; writing—review and editing, all authors; visualization, A.K. and B.L.; supervision, T.R., B.L., A.L. and L.S.; project administration, B.L. and T.R.; funding acquisition, T.R. and B.L. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data analyzed or generated during this study are included in the manuscript.

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4.3. Manuscript 3: New Emergence of the Novel Pestivirus Linda Virus in a Pig Farm in Carinthia, Austria

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New Emergence of the Novel Pestivirus Linda Virus in a Pig Farm in Carinthia, Austria

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Abstract: Linda virus (LindaV) was first identified in a pig farm in Styria, Austria in 2015 and associated with congenital tremor (CT) type A-II in newborn piglets. Since then, only one more LindaV affected farm was retrospectively discovered 10 km away from the initially affected farm. Here, we report the recent outbreak of a novel LindaV strain in a farrow-to-finish farm in the federal state Carinthia, Austria. No connection between this farm and the previously affected farms could be discovered. The outbreak was characterized by severe CT cases in several litters and high preweaning mortality. A herd visit two months after the onset of clinical symptoms followed by a diagnostic workup revealed the presence of several viremic six-week-old nursery pigs. These animals shed large amounts of virus via feces and saliva, implying an important epidemiological role for within- and between-herd virus transmission. The novel LindaV strain was isolated and genetically characterized. The findings underline a low prevalence of LindaV in the Austrian pig population and highlight the threat when introduced into a pig herd. Furthermore, the results urge the need to better understand the routes of persistence and transmission of this enigmatic pestivirus in the pig population.

Keywords: emerging disease; Flaviviridae; pestivirus; atypical porcine pestivirus; Bungowannah virus; congenital tremor; Linda virus; novel Linda virus strain; viruses; Austria

1. Introduction

The genus *Pestivirus* within the family *Flaviviridae* includes economically important pathogens of pigs. In addition to the OIE listed classical swine fever virus (CSFV, *Pestivirus C*) [1], Bungowannah virus (BungoV, *Pestivirus F*), atypical porcine pestivirus (APPV, *Pestivirus K*), and Linda virus (LindaV, *Pestivirus L*) have been detected in swine [2–4]. BungoV emerged in 2003 and is responsible for reproductive disorders, the birth of stillborn and mummified piglets, and sudden death in weaning age piglets [2]. A single outbreak of BungoV was detected in Australia and contained, but the virus has not yet been eradicated [5]. A spread of BungoV has been considered a threat to global porcine health, but there is no evidence to date that BungoV has established itself in other regions of the

world [6–9]. In contrast, APPV occurs worldwide and is responsible for congenital tremor (CT) type A-II in piglets causing moderate economic losses [10–14].

LindaV was discovered as a novel "lateral-shaking inducing neurodegenerative agent" in a piglet-producing farm in Styria, Austria in 2015 [4]. Piglets infected in utero showed a severe lateral shaking of the whole body, causing incapability of sucking milk, which led to a high preweaning mortality [4]. CT type A-II was confirmed during histopathological examinations of diseased piglets with the presence of severe hypomyelination in the spinal cord and of viral antigen in tissues of the central nervous system (CNS) [4]. Experimental LindaV infections of immunocompetent piglets have been found to result in transient viremia and rapid seroconversion [15]. However, the virus persisted in lymphoid tissues and was still detectable after 21 days. To date, LindaV has been detected in the index case as well as in a farm 10 km away as a consequence of a nationwide screening for LindaV neutralizing antibodies [16]. This study revealed a very low seroprevalence of 0.15% (based on the number of sera screened) and 0.7% (at farm level) in Austria [16]. A novel, genetically closely related LindaV strain (LindaV strain Austria2) could be isolated from a serum sample in 2016 [16]. Interestingly, clinical signs of CT had never occurred in pigs of that farm, according to the farmer and responsible veterinarian.

In this study, we present the isolation of a novel LindaV strain (LindaV strain Austria3) causing clinically relevant disease in a farrow-to-finish farm in Carinthia, Austria in 2020/2021.

2. Materials and Methods

2.1. Farm Description

A commercial farrow-to-finish farm in Carinthia, Austria produces piglets with 60 Large White and Landrace crossbred sows in a continuous farrowing cycle. One Pietrain boar is used for semen production, natural insemination, and for sexual stimulation of sows, which are inseminated artificially. Piglets are weaned at 28 days of age. The piglets are routinely vaccinated against *Mycoplasma hyopneumoniae* and against porcine circovirus 2 (PCV2) using inactivated vaccines (*M. hyopneumoniae* in the first week of life with Suvaxyn® MH-One, Zoetis Österreich GmbH, Vienna, Austria; PCV2 at 21 days of age with Suvaxyn Circo, Louvain-la-Neuve, Belgium).

Before onset of the reproductive disorders, new gilts were purchased in March 2020 from a commercial gilt producer and introduced into the herd after an isolation phase of eight weeks. Gilts and sows are immunized against parvovirosis and erysipelas using a combined inactivated vaccine (Parvoruvac, Ceva Santé Animale, Libourne, France) in the isolation unit and at the time point of weaning, respectively. Gilts and sows are vaccinated against porcine reproductive and respiratory syndrome virus (PRRSV) using a modified live virus vaccine (Porcilis PRRS, Intervet GesmbH, Vienna, Austria) in a five-month interval. Vaccination against swine influenza A virus using a trivalent vaccine was introduced after the reproductive disorders started.

2.2. Diagnostic Samples

The samples were obtained by veterinarians of the University Clinic for Swine (University of Veterinary Medicine, Vienna) for diagnostic purposes during a farm visit. Therefore, no ethical approval was needed for this study. A one-day-old non-viable piglet and an eight-week-old nursery pig with a paralysis of both hind legs were humanely euthanized under general anesthesia (anesthesia: 1.3 mg/kg azaperone and 10 mg/kg ketamine hydrochloride; euthanasia: T61 (5.0 mg/mL tetracaine hydrochloride, 50 mg/mL mebezonium iodide and 200 mg/mL embutramide; 1 mL/10 kg)). The following tissues were collected from the euthanized piglets: parotid gland, tonsil, lymph node (inguinal, tracheobronchial and mesenterial), heart, lung, liver, pancreas, duodenum, jejunum, ileum, caecum, colon, kidney, genital tract, urinary bladder, thymus, spleen, cerebellum, medulla oblongata, spinal ganglion, spinal cord, sciatic nerve, and umbilical cord (only newborn piglet). EDTA-treated blood and whole blood samples were drawn from two sows with previous CT litters,

15 nursery pigs and 2 euthanized piglets as mentioned above (euthanized eight-week-old nursery pig only EDTA-treated blood sample). Pooled fecal samples were collected from the gestation barn, farrowing crates, and the flat deck. Semen and a saliva swab sample were obtained from the boar. Further saliva swabs were collected from 12 sows and gilts and 15 nursery pigs (nursery pigs pooled swab sample). A pooled placenta sample from newly farrowed sows and tails from newborn piglets were also obtained.

Additionally, paraffin-embedded tissue samples (brain, liver, spleen, kidney, gastrointestinal tract, lung, and heart) originating from a CT affected piglet were kindly provided by the veterinary diagnostic laboratory of the federal state, Carinthia. These tissue samples were obtained during the acute congenital tremor phase in December 2020.

2.3. Pathological Examination and Immunohistochemistry

A full necropsy was performed on the eight-week-old nursery pig, euthanized because of a paralysis of both hind legs. Samples of brain, spinal cord, peripheral nerves, liver, spleen, kidneys, gastrointestinal tract, lung, lymph nodes, tonsils, thymus, pancreas, and salivary glands were fixed in 4% buffered formalin and embedded in paraffin wax. Two µm thick sections were cut and stained with hematoxylin and eosin (HE). Selected brain and spinal cord samples were stained with a combination of luxol fast blue and HE to assess the myelin content. Furthermore, paraffin-embedded samples (brain, liver, spleen, kidney, gastrointestinal tract, lung, and heart) from one CT affected piglet, which had been confirmed to be positive for LindaV by RT-PCR, were stained with HE.

CNS samples of both pigs were evaluated by immunohistochemistry using a primary anti-pestivirus E2 antibody (mouse monoclonal antibody (mAb) 6A5, dilution 1:100) for the detection of LindaV. Stainings were performed automatically on an autostainer (Lab Vision AS 360, Thermo Fisher Scientific, Waltham, MA, USA). Antigen retrieval was performed by pronase digestion. After application of the primary antibody, a polymer detection system (UltraVision LP Large Volume Detection System; Thermo Fisher Scientific, Waltham, MA, USA), consisting of a universal secondary antibody formulation conjugated to an enzyme-labeled polymer was used. The polymer complex was then visualized with diaminobenzidine (Labvision/Thermo Fisher Scientific, Waltham, MA, USA). Sections were counterstained with hematoxylin. Formalin fixed, paraffin-embedded spinal ganglia of pigs from the previous outbreak of LindaV in 2015 served as positive and negative controls, respectively.

2.4. Peripheral Blood Mononuclear Cell (PBMC) Isolation

EDTA-treated blood was centrifuged for 10 min at 2000× *g* and 4 °C. Plasma was collected from the supernatant and stored at −20 °C. The buffy coat layer was carefully removed (approximate volume 1 mL), transferred to a centrifuge tube containing 5 mL erythrocyte lysis buffer (Buffer EL, QIAGEN, Hilden, Germany) and thoroughly vortexed. The mixture was incubated for 10 min at 4 °C, followed by a centrifugation step for 10 min at 1000× *g* and 4 °C. The supernatant was removed and the procedure was repeated twice. Finally, the PBMC pellet was resuspended in 1 mL Dulbecco's modified Eagle's medium (DMEM, Biowest, Nuaillé, France).

2.5. Cell Culture

SK-6 cells [17] were cultured in DMEM (Biowest) supplemented with 10% heat-inactivated fetal calf serum (FCS, Corning, Tewksbury, MA, USA; tested negative for pestiviruses), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37 °C and 5% CO_2.

2.6. Indirect Immunofluorescence Assay

The indirect immunofluorescence assay was performed as previously described [4]. Briefly, SK-6 cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C and permeabilized with 1% Triton-X 100 (Merck, Darmstadt, Germany) in PBS for 5 min at

room temperature. The cross-reactive mAb 6A5 (anti-BVDV E2) was used as a primary antibody and a goat anti-mouse IgG conjugated with Cy3 (Dianova, Hamburg, Germany) was used as a secondary antibody.

2.7. Virus Isolation

A total volume of 50 µL PBMCs resuspended in DMEM (as described in Section 2.4) was co-cultured with 5×10^4 SK-6 cells on a 24-well cell culture plate (STARLAB, Hamburg, Germany). Cells and cell culture supernatant were passaged 72 h post co-cultivation. Successful infection of SK-6 cells was detected using an indirect immunofluorescence assay (as described in Section 2.6) and a LindaV-specific RT-qPCR assay (as described in Section 2.9).

2.8. Serum Virus Neutralization (SVN) Assay

The SVN assay was performed as previously described [16]. Briefly, sera were heat inactivated at 56 °C for 30 min and a five-fold serial dilution (1/5 starting dilution, 1/390,625 final dilution) was prepared in DMEM (Biowest) in a 96-well cell culture plate (STARLAB). An mCherry-labeled LindaV stock (1.78×10^5 $TCID_{50}$/mL) was diluted to 100 $TCID_{50}$/50 µL, added to the serum dilutions and incubated for 2 h. A total number of 1×10^4 SK-6 cells was added to the serum/virus-mixture and further incubated for 72 h. Serum controls, cell controls, positive and negative reference antisera, and a virus back titration were included. Cells were fixed with 4% paraformaldehyde and evaluated using a fluorescence microscope (Olympus IX70 fluorescence microscope; OLYMPUS, Hamburg, Germany).

The 50% neutralization dose (ND_{50}/mL) was calculated based on the Spearman–Kaerber method and expressed as the reciprocal value (1/ND_{50}/mL) of the serum dilution.

2.9. RNA Extraction and LindaV-Specific RT-qPCR Assay

Total RNA extraction was performed using the QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions. A total volume of 140 µL serum, plasma, or semen was directly used for RNA extraction. Saliva swab samples were moistened with 1 mL PBS, mixed by vortexing, then were centrifuged and 140 µL supernatant was used for RNA extraction. Each tissue sample of 100 mg was mixed with 1 mL PBS in a 2 mL microcentrifuge tube containing stainless steel beads and homogenized at a frequency of 30/s for 3 min using a TissueLyser II (QIAGEN). After centrifugation, a volume of 140 µL homogenate supernatant was used for extraction. PBMCs were lysed by three freeze–thaw cycles, cell debris was removed by centrifugation, and 140 µL supernatant were used for RNA extraction.

For the detection of LindaV RNA, a LindaV-specific RT-qPCR assay was performed on a Rotor-Gene Q cycler (QIAGEN) using the Luna Universal Probe One-Step RT-qPCR Kit (NEB, Ipswich, Massachusetts, United States) as previously described [15]. A beta-actin RT-qPCR assay was used as an internal control, as previously described [16] under the same cycling conditions as the LindaV RT-qPCR assay. Saliva swab samples were spiked with a plasmid harboring the enhanced green fluorescent protein (EGFP) coding sequence prior to RNA extraction to exclude inhibitory factors in the RT-qPCR run, as the beta-actin RT-qPCR yields inconsistent results in the analysis of this sample material. The oligonucleotides EGFP-F (5′-GACCACTACCAGCAGAACAC-3′), EGFP-R (5′-GAACTCCAGCAGGACCATG-3′), and the probe EGFP-HEX (5′-HEX-AGCACCCAGTCCGCCCTGAGCA-BHQ-1-3′) were used for the amplification of a 132 bp fragment of the EGFP sequence, as described by Hoffmann et al. [18], under the same cycling conditions as the LindaV RT-qPCR assay.

A ten-fold dilution series of a plasmid harboring the target sequence for the LindaV RT-qPCR assay was included in each run to obtain a standard curve for quantification of the viral load in the samples, as previously described [15]. Briefly, the copy number per reaction was estimated by the Rotor-Gene Q software (version 2.3.4.; QIAGEN) based on the copy number of the input plasmid DNA and converted to the respective genome equivalents of each sample (GE/mL, GE/g, or GE/swab). Since we used a DNA standard and not an

RNA standard in our RT-qPCR assay, efficiency of the reverse transcription step could not be assessed, which could lead to minor deviations in the copy number calculations.

2.10. RT-PCRs, Sanger Sequencing and Sequence Analysis

A pan-pestivirus RT-PCR amplifying a fragment of the NS5B coding region was conducted using the OneTaq One-Step RT-PCR Kit (NEB) and oligonucleotides as previously described [4]. The following cycling conditions were used: 48 °C for 20 min, 94 °C for 1 min, followed by 45 cycles of 94 °C for 15 s, 50 °C for 30 s, 68 °C for 1:10 min, and a final elongation at 68 °C for 5 min. The amplicon with an approximate length of 800 bp was purified using the Quantum Prep PCR Kleen Spin Columns (Bio-Rad, Hercules, CA, USA) and the sequence of the purified PCR product was determined by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). The obtained sequence was analyzed using the Nucleotide Basic Local Alignment Search Tool (BLASTN) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, accessed on 4 June 2021).

The full genome of the novel LindaV strain Austria3 (GenBank accession number: OK086026) was obtained by a two-step RT-PCR approach with the generation of overlapping PCR fragments using oligonucleotides and cycling conditions as previously described [16]. The PCR products were purified using the Quantum Prep PCR Kleen Spin Columns (Bio-Rad) and the sequences were determined by Sanger sequencing (Eurofins Genomics). For the determination of the full-genomic sequence of the novel LindaV strain, a consensus sequence missing only the ultimate 5′- and 3′-ends was generated and compared to the available LindaV sequences (GenBank accession numbers: KY436034.1 (LindaV strain Austria1) and MZ027894.1 (LindaV strain Austria2)). Sequence analysis was performed using the DNA Strider 3.0 Software [19,20] and CLC Sequence Viewer 7.7.1 (CLC bio/QIAGEN Digital Insights, Aarhus, Denmark).

2.11. Phylogenetic Analysis

Phylogenetic analysis was performed using the CLC Sequence Viewer 7.7.1 (CLC bio/QIAGEN Digital Insights, Aarhus, Denmark) based on the complete genome sequences. The neighbor-joining algorithm and bootstrapping with 1000 replicates were used for the construction of the phylogenetic tree. The following pestivirus sequences were used for the analysis: Linda virus strain Austria1 (KY436034.1, *Pestivirus L*), Linda virus strain Austria2 (MZ027894.1), Linda virus strain Austria3 (OK086026), Bungowannah virus (EF100713.2, *Pestivirus F*), Dongyang pangolin pestivirus isolate DYAJ1 (MK636874.1, *Pestivirus P*), Phocoena pestivirus isolate NS170386 (MK910229.1, *Pestivirus M*), atypical porcine pestivirus 1 strain AUT-2016_C (KX778724.1, *Pestivirus K*), and classical swine fever virus strain Alfort_187 (X87939.1, *Pestivirus C*).

3. Results

3.1. Description of the Novel LindaV Outbreak

In autumn 2020, a farrow-to-finish farm in the federal state Carinthia in the south of Austria (Figure 1) reported cases of reproductive disorders (abortions, neonatal deaths, birth of stillborn and mummified piglets) in several sows and gilts. The symptoms started in October 2020, when four sows farrowed four weeks prior to the calculated farrowing date. These piglets were born alive, but died soon after birth. The subsequent seven litters presented with symptoms known for parvovirosis: mummies in different developmental stages, stillborn, and live-born piglets. The episode of reproductive failure was followed by the occurrence of severe CT in a total of 20 litters and associated with a high preweaning mortality of 80–90% in December 2020 (Video S1). The last CT affected litters occurred at the beginning of January 2021. During the CT episode in the farm, approximately 10–20% of piglets were born weak, and the return-to-estrus rate was 10%. The number of weaned piglets per sow and year dropped from an average of 28 to an average of 22 due to reproductive disorders and piglet mortality. Pooled organ samples from five CT affected

piglets were sent to the University of Veterinary Medicine, Vienna in mid-January 2021. The analysis of the organ samples using a pan-pestivirus RT-PCR in the NS5B region yielded a positive result. In the diagnostic laboratory of the Institute of Virology at the University of Veterinary Medicine, Vienna, PRRSV and porcine parvovirus (PPV) were ruled out by PCR as differential diagnoses, and results of a PCV2 qPCR were below the level of quantification. Sequencing of the pestiviral NS5B amplicon with a length of approximately 800 bp and analysis of the consensus sequence using Nucleotide Basic Local Alignment Search Tool (BLASTN) revealed a sequence identity of 98.3% to the LindaV strain Austria1 found in 2015 (GenBank accession number: KY436034.1). The pooled organ samples were re-assayed using a LindaV-specific RT-qPCR in the 5′-UTR region, which determined a very high viral load of 3.9×10^8 GE/g.

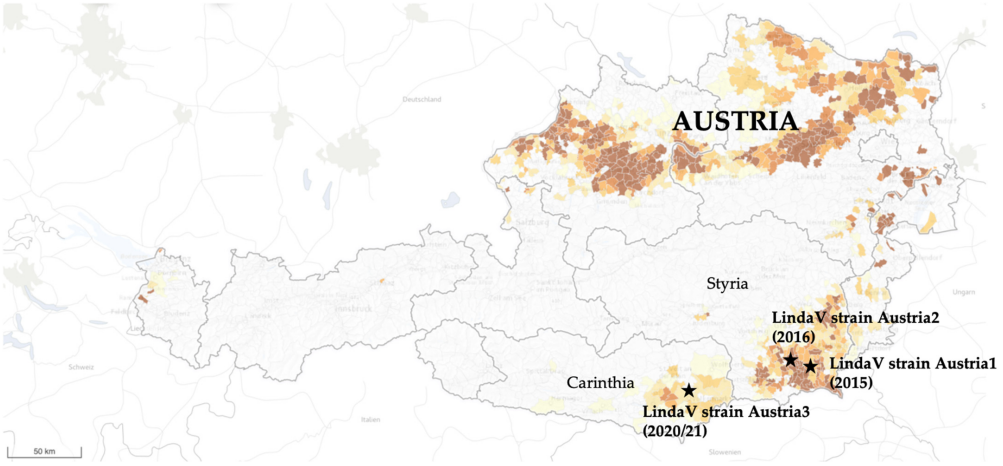


Figure 1. Locations of the farms where Linda virus (LindaV) strains Austria1, Austria2, and the novel strain Austria3 were isolated. Stars indicate the locations of the farms. Different brown shades mark regions with a high (dark brown) and a low (light brown) pig density. LindaV: Linda virus. (Modified from: https://www.statistik.at/atlas/?mapid=them_lw_as2010_viehbetriebe&layerid=layer1&sublayerid=sublayer0&languageid=0 (accessed on 18 August 2021). © Statistics Austria—Cartography and GIS, created 1 September 2018).

Subsequently, a farm visit was conducted in February 2021 for a diagnostic workup of the disease outbreak. At this time, no clinical symptoms associated with LindaV infection were observable and reproduction data approached pre-outbreak levels. Two sows, which farrowed the day before the farm visit, presented with homogenous litters of clinically healthy newborn piglets. All other sows and suckling piglets in the farrowing barn appeared clinically healthy. A batch of six-week-old nursery pigs surviving the CT phase presented as a heterogenous group with ill-thrift and runting piglets. An eight-week-old nursery pig suffering from a paralysis of both hind legs was observed and humanely euthanized. All sows and the boar appeared clinically healthy, whilst mild dry coughing was noticed in finisher pigs.

3.2. Diagnostic Workup

3.2.1. Serum Virus Neutralization Assay

Sera of 15 six-week-old nursery pigs (P1–P15), plasma of an eight-week-old nursery pig, sera of two sows with previous CT litters, and serum of a one-day-old non-viable piglet were analyzed in the SVN assay (as described in Section 2.8).

High neutralizing antibody titers ≥1/193.2 ND_{50}/mL were detectable in nine nursery pigs (P1, P5, P7–P9, and P12–P15). Intermediate to low neutralizing antibody titers between 1/17.2 ND_{50}/mL and 1/86.4 ND_{50}/mL were found in four animals (P2, P6, P10, and P11). Two nursery pigs (P3 and P4) did not show any neutralizing activity (Figure 2). A high neutralizing activity of 1/10,640 ND_{50}/mL was determined in the plasma of the eight-week-old nursery pig, euthanized because of a paralysis of both hind legs. Two sows with previous CT litters showed a strong neutralizing activity against LindaV (both 1/2180 ND_{50}/mL). No neutralizing activity was detectable in a one-day-old, non-viable piglet.

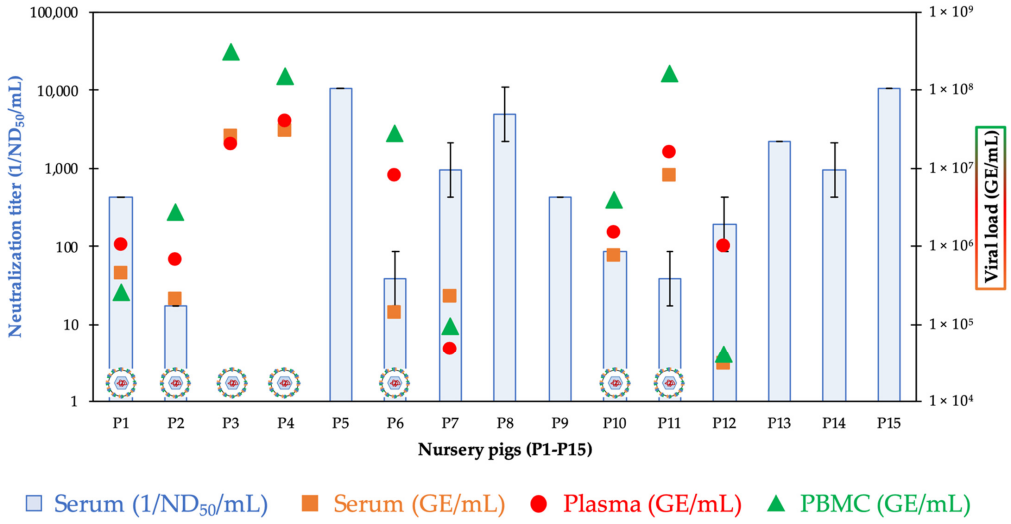


Figure 2. Results of the serum virus neutralization (SVN) assay (ND_{50}/mL), the LindaV-specific RT-qPCR assay from serum, plasma and PBMCs (GE/mL), and virus isolation from PBMCs of six-week-old nursery pigs (P1–P15). Virus particles represent successful virus isolation through co-culturing of PBMCs with SK-6 cells. Neutralizing antibody titers are given as the reciprocal ND_{50} value and error bars indicate positive and negative standard deviations. ND_{50}, 50% neutralization dose; GE, genome equivalents; PBMC, peripheral blood mononuclear cell.

3.2.2. LindaV-Specific RT-qPCR Assay

The LindaV-specific RT-qPCR assay and the RT-qPCR assays for the internal control genes beta-actin and eGFP were performed as described in Section 2.9. All samples analyzed in the LindaV RT-qPCR assay were positive in the RT-qPCR assays for the internal control genes beta-actin and eGFP.

The results of the LindaV-specific RT-qPCR assay of the six-week-old nursery pigs (P1–P15; serum, plasma, and PBMCs) are shown in Figure 2 and described in detail in Supplementary Table S1. The analysis of a pooled saliva swab sample and a pooled fecal sample from the six-week-old nursery pigs (P1–P15) confirmed viral shedding, showing a high viral load of 1.17×10^6 GE/swab (pooled saliva swab sample) and 2.65×10^6 GE/g (pooled fecal sample) (Supplementary Table S1).

Furthermore, tissue samples from different organs and blood samples (plasma and PBMCs) of an eight-week-old nursery pig, euthanized because of a paralysis of both hind legs, were analyzed in the RT-qPCR assay. LindaV RNA was detected in lymphoid organs (inguinal lymph node and tonsil) and tissues of the CNS (cerebellum and medulla oblongata) and the peripheral nervous system (PNS; spinal ganglion). The viral loads ranged from 2.25×10^5 GE/g to 5.36×10^6 GE/g (Supplementary Table S2). All other tissue samples tested negative by RT-qPCR and viremia was not detectable in this animal.

LindaV RNA was not detectable in any of the samples obtained from sows and gilts (feces, saliva swab samples, and blood samples). All samples obtained from the boar, the newborn piglets, and the pooled placenta sample were negative in the LindaV-specific RT-qPCR.

3.2.3. Virus Isolation

Virus isolation was conducted through the co-culturing of PBMCs with susceptible SK-6 cells (as described in Section 2.7). Virus isolation was successful in 7 out of 15 nursery pigs (P1–P4, P6, P10, and P11) (Figure 2 and Supplementary Table S1). No virus could be isolated from PBMCs of the sows and the euthanized one-day-old piglet.

3.2.4. Histopathology and Immunohistochemistry

Paraffin-embedded samples of cerebellum and brainstem from one CT piglet were available for histological and immunohistochemical evaluation (as described in Section 2.3). HE staining showed no lesions, but LindaV could be detected in the cytoplasm of neurons by immunohistochemistry (Figure 3).

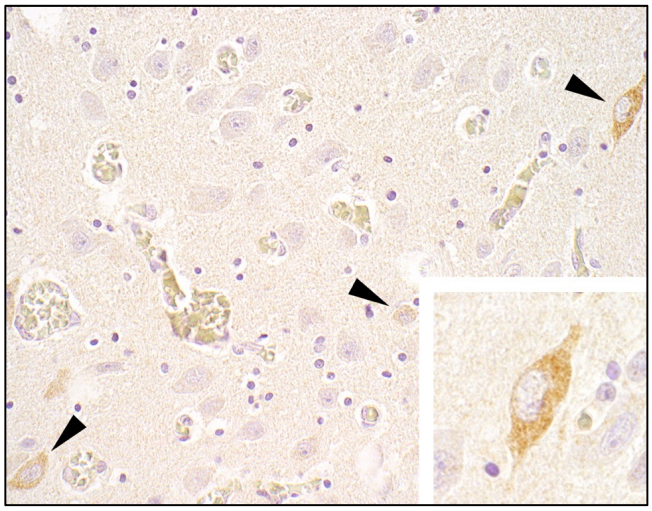


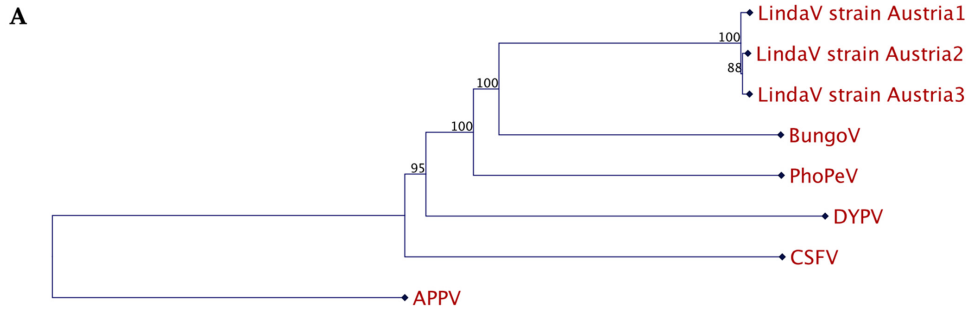
Figure 3. Detection of LindaV by immunohistochemistry in the cytoplasm of neurons (arrowheads) in the brainstem of a CT piglet. Primary antibody: pestivirus E2-specific monoclonal antibody 6A5; counterstain: hematoxylin, magnification 400×, insert 600×.

Furthermore, tissue samples of the eight-week-old nursery pig were analyzed. Immunohistochemistry for the detection of LindaV showed no positive results in any of the tissues. While determining cause of the paralysis of both hind legs, randomly distributed foci of neuronal necrosis in the gray matter and degeneration of the white matter of the caudal cervical spinal cord with very mild reactive changes and scattered small hemorrhages were detected. The etiology of these lesions could not be revealed. Multifocal scattered perivascular lymphoplasmacellular infiltrations were present in the brain and spinal cord.

3.3. Genetic Characterization of the Novel LindaV Strain Austria3

The full genome sequence of the novel LindaV strain Austria3 (OK086026) was obtained using a two-step RT-PCR approach and Sanger sequencing. We chose the sample with the highest viral load in the RT-qPCR assay (3.16×10^8 GE/mL; PBMCs of nursery pig P3) for this sequencing attempt. A consensus sequence of 12,568 nt was determined and compared with the sequences of LindaV strain Austria1 (KY436034.1) and LindaV

strain Austria2 (MZ027894.1) (Figure 4). Interestingly, LindaV strain Austria2 and LindaV strain Austria3 were found to be slightly more closely related than they were to the index case LindaV strain Austria1; the total nucleic acid sequence identity was 98.9% compared to 98.5%, respectively. The sequence identities between the different coding regions of the LindaV genome are shown in Figure 4B. The divergence in the nucleic acid sequence resulted in a comparable total amino acid identity of 98.3% between both LindaV strain Austria1 and LindaV strain Austria2 as well as LindaV strain Austria1 and LindaV strain Austria3. In contrast, total amino acid identity was 99.1% when comparing LindaV strain Austria2 and LindaV strain Austria3.



B

Identity between LindaV strains (in %)		5′UTR	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	3′UTR
	LindaV strains Austria1/2	99.4	98.1	99.3	99.0	98.4	97.8	98.6	98.2	98.5	98.4	98.9	98.5	98.5	98.5
	LindaV strains Austria1/3	98.9	98.7	99.6	98.7	99.1	97.7	98.6	98.0	98.9	98.4	98.0	98.8	98.3	98.6
	LindaV strains Austria2/3	99.4	98.7	99.6	99.0	98.6	98.4	99.5	98.6	99.0	98.9	98.7	98.9	99.3	98.5

Figure 4. Phylogenetic analysis of selected pestivirus species and sequence analysis of the full-genomic sequences of the so far known Linda virus strains. (**A**) An unrooted phylogenetic tree was constructed based on the full-genomic sequences of LindaV strain Austria1, LindaV strain Austria2, LindaV strain Austria3, Bungowannah virus, Phocoena pestivirus isolate NS170386, Dongyang pangolin pestivirus isolate DYAJ, CSFV strain Alfort_187, and APPV strain AUT-2016_C using the neighbor-joining algorithm and bootstrapping with 1000 replicates. Bootstrap values are shown in percentage at each node. (**B**) Sequence identities between the different coding regions of the viral genome of LindaV strains Austria1, Austria2, and Austria3 are given in percentage. The coding regions for the nonstructural proteins are highlighted in dark green and coding regions for the structural proteins are in light green. DYPV, Dongyang pangolin pestivirus; PhoPeV, Phocoena pestivirus; BungoV, Bungowannah virus; APPV, atypical porcine pestivirus; CSFV, classical swine fever virus; UTR, untranslated region; C, Core; E, envelope glycoprotein; NS, nonstructural protein.

4. Discussion

The discovery of LindaV in Styria, Austria in 2015 expanded the group of pestiviruses infecting the host species swine and revealed the long-sought European relative to BungoV from Australia [4]. Despite an elevated level of attention after the first LindaV outbreak, neither signs of viral spread nor the existence of this virus in other pig herds have been observed [8,12]. Nevertheless, LindaV was found in a farm a 10 km distance from the index case by retrospective sero-surveillance without clinical indications [16]. In this paper, we describe a new outbreak of LindaV that occurred six years later in another province

of Austria. Similar to the index case, CT and high preweaning mortality occurred in the piglets following reproductive disorders in the sows. Furthermore, LindaV was detectable in six-week-old pigs, which suffered from CT as newborn piglets, suggesting a chronic or persistent infection in those animals.

Intrauterine infections with pestiviruses at early stages of gestation may cause immunotolerance [21–25]. Immunotolerant animals become persistently infected (PI) and shed large amounts of virus throughout their lifespan. After intrauterine infection of the fetus with BungoV, a long-lasting, high-level viremia above 10^6 GE/mL up to 75 days of age has been observed [25]. While studies assessing the outcome of fetal infection with LindaV are still missing, the high-level viremia of up to 3×10^8 GE/mL together with the lack of humoral immune response against LindaV in individual animals (piglets P3 and P4) indicate the occurrence of such chronically, possibly persistently infected animals. Furthermore, these six-week-old animals shed large amounts of virus via the fecal and oral route, supporting this hypothesis.

High titers of neutralizing antibodies were found in some viremic piglets, which could have been passively acquired by colostrum ingestion. Unfortunately, no discrimination between maternally-derived and actively acquired antibodies was possible in our test. Titers of maternally-derived antibodies can last for a long time after pestiviral infection. Maternally-derived antibodies against BVDV have been detected around 190 days [26] and against CSFV over a period of seven weeks [27]. Therefore, the presence of maternal antibodies cannot be excluded.

It has been shown that PBMCs represent a reliable specimen for the detection of the cell-associated viremia of BVDV [28] and CSFV [29]. In this study, we detected high viral loads of LindaV RNA in PBMCs and isolated the virus through co-culturing with SK-6 cells. Several highly viremic animals showed a 10-fold higher viral load in PBMCs compared to serum and plasma, suggesting that the analysis of PBMCs represents a more sensitive detection method for the viremic phase of LindaV infections. Virus isolation from PBMCs was successful starting from 2.58×10^5 GE/mL, even though a high neutralizing antibody titer of 1/434 ND_{50}/mL was observed in one animal (P1). In this case, virus isolation attempts from serum or plasma could lead to false negative results due to circulating neutralizing antibodies.

In the eight-week-old nursery pig suffering from a paralysis of both hind legs, focally extensive acute lesions in gray and white matter in the caudal cervical spinal cord were present with only mild reaction. Neither the distribution nor the type of lesions were representative of a viral infection. Therefore, the lesions were thought to be most likely of ischemic or traumatic origin. Unrelated to these lesions, multifocal scattered perivascular lymphoplasmacellular infiltrations were detected in the brain and spinal cord, which might be due to an infection with LindaV, which is in accordance with the viral RNA detected in structures of the CNS by RT-qPCR. The negative results in the immunohistochemical analysis could indicate a lower sensitivity of this technique compared to the RT-qPCR assay.

There is high sequence identity between all three currently known LindaV strains (Austria1–Austria3). We found no evidence for transmission or epidemiological links between the cases in Styria and the new outbreak in Carinthia. Therefore, we must assume a low but steady prevalence of LindaV in Southern Austria. The purchase of new gilts from a commercial gilt producer and the disease outbreak approximately six months after their introduction into the pig herd may be a possible route of transmission. Investigations of the presence of LindaV in the gilt-producing facility are underway. However, gilt-producing farms in Austria usually sell pigs to several pig holdings and a single disease outbreak would be unlikely considering the low seroprevalence of LindaV infections in the Austrian pig population [16]. Since our seroprevalence study could not detect reservoirs in domestic pig herds, it is reasonable to postulate a reservoir host in the wild. Wild boar and wild ruminant species could represent the reservoir of LindaV. Because pestiviruses have also been described in the recent past in some non-cloven-hoofed animals [30–33], other wildlife reservoirs, such as rodents or bats, cannot be excluded. To prevent further introduction

of the virus into the domestic pig population, identification of reservoir hosts is critical. A steady surveillance should also be established in the risk region of Southern Austria to prevent spread via clinically inconspicuous, long-term viremic animals.

5. Patents

The authors B.L., L.S., and T.R. are inventors of a patent on LindaV pestivirus (PCT/EP2017/084453; Isolation of a novel pestivirus causing congenital tremor).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14020326/s1, Video S1: Piglets affected with congenital tremor (CT), Supplementary Table S1: Results of serum virus neutralization (SVN) assay (1/ND_{50}/mL), RT-qPCR assay (GE/mL; GE/g; GE/swab) and virus isolation from six-week-old nursery pigs, Supplementary Table S2: Results of SVN assay (1/ND_{50}/mL), RT-qPCR assay (GE/mL, GE/g) and virus isolation from an eight-week-old euthanized nursery pig suffering from a paralysis of both hind legs.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, because the samples were taken by authorized veterinarians in frame of a farm visit for diagnostic purposes.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data analyzed or generated during this study are included in the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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5. Discussion & Conclusion

LindaV was discovered as a novel pestivirus in an Austrian pig farm in 2015. A far distant genetic relationship to other pestiviruses, cross-reactivity of an anti-pestivirus E2 antibody and a rapid, high titer propagation in porcine cell lines were subsequently demonstrated. Interestingly, LindaV is the closest genetic relative of BungoV from Australia, but affected piglets suffered from CT type A-II, which is reminiscent of APPV infections in newborn piglets. One of the main goals of our study was to get more insights into the epidemiological situation of LindaV in the Austrian domestic pig population, as there were no data available about its occurrence and no further disease outbreaks were reported. Another aim was to assess the virulence of LindaV, the efficiency of viral replication and the immune response in acutely infected immunocompetent pigs.

We assessed the virulence of LindaV in an acute infection experiment of twelve-week-old, immunocompetent post-weaning piglets. Similar to acute infections with BungoV (Finlaison et al., 2012) or low virulent strains of CSFV (Weesendorp et al., 2009), infected animals did not show any or only mild clinical signs of disease, viral replication was low and viremia was hardly detectable. We can assume that LindaV is a benign virus for pigs with a fully developed immune system, similar to BungoV (Finlaison et al., 2012), independent of the infection route (intranasally or intramuscularly). Despite the low replication rate, transiently infected animals successfully transmitted the virus to naïve sentinel pigs, but to a very low extent. Therefore, animals acutely infected with LindaV are most likely not the major source for virus transmission in a pig herd, as was already demonstrated for BVDV (Houe, 1995). Nevertheless, acutely infected animals developed a strong humoral immune response with high neutralizing antibody titers against LindaV, starting between 7 to 14 days post infection and reaching peak titers around 21 days post infection. Seroconversion and a strong neutralizing activity were also observed in animals acutely infected with BungoV (Finlaison et al., 2012). These findings are in accordance with the development of humoral immunity against the classical pestiviruses, e.g. BVDV. Here, high neutralizing antibody titers have been detected after 14 to 21 days post infection, which last for several years or even lifelong (Fredriksen et al., 1999). The presence of high-level neutralizing antibodies against LindaV was experimentally shown until 28 days post infection, but serological data from sows of the index farm in Styria indicate a persistence of high neutralizing antibody titers for at least two years after natural infection (unpublished).

The acute infection experiment provided valuable first results on the biology of LindaV. Nevertheless, a controlled infection study of pregnant sows should be conducted in the future to experimentally reproduce the clinical disease in newborn piglets observed in the field after diaplacental infection with LindaV. Successful transplacental infection with post-natal disease symptoms was already demonstrated for the recently discovered atypical porcine pestiviruses APPV (Arruda et al., 2016; De Groof et al., 2016) and BungoV (Finlaison et al., 2010; Finlaison and Kirkland, 2020a, 2020b). Moreover, Finlaison et al. determined the critical stage for the development of persistent infections with BungoV to approximately day 35 to 55 of gestation (Finlaison and Kirkland, 2020a).

Important parameters for the classification and demarcation of novel pestivirus species are the genetic and antigenic relatedness, and the host of origin (https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae/361/genus-pestivirus, accessed 2022 January, 31). A far distant genetic relationship of LindaV to the other pestivirus species was already demonstrated by Lamp et al., with a nucleotide and amino acid sequence identity of approximately 60 % to the classical pestiviruses and APPV, and approximately 70 % to BungoV (Lamp et al., 2017). The antigenic relationship can be determined by monoclonal antibody binding assays or cross-neutralization assays (Paton et al., 1995). The LindaV anti-sera, obtained from the acute infection experiment, enabled us to study the antigenic relationship in cross-neutralization assays. The absence of cross-neutralization to all of the tested pestivirus species highlights the far distant relationship and strongly indicates the classification as a separate pestivirus species. Based on these data and further phylogenetic analyses, LindaV was officially proposed as a novel pestivirus species, *Pestivirus L* (Postel et al., 2021).
The co-existence and co-circulation of novel pestiviruses could hamper the diagnostic methods for the detection of the notifiable pestiviral diseases CSF or BVD, due to cross-reactivities in diagnostic assays. The interference of APPV with detection methods for CSFV was already excluded by Postel et al. (Postel et al., 2017b). According to the results of our cross-neutralization assays, cross-reactivity of LindaV with other pestiviral serological assays seems rather unlikely, too.

The outcome of the acute infection experiment facilitated the design of our epidemiological study. We observed a short-term, low-level viremia in acutely infected animals, which is hardly detectable despite highly sensitive molecular methods. Together with the presumably low

prevalence of persistently infected animals, as was demonstrated for other pestiviruses, e.g. BVDV (Houe, 1995), the chances to detect LindaV RNA in the pig population were considered very low. On the contrary, rapid seroconversion with presumably long-lasting high levels of neutralizing antibody titers were detected in acutely infected animals, thus the SVN assay was used for our epidemiological study. Compared to other serological assays, e.g. different ELISA systems or immunofluorescence assays, the SVN assay shows the highest sensitivity and specificity in the serologic diagnosis of pestiviral infections, especially in combination with cross-neutralization assays (World Organisation for Animal Health, 2019b, 2019a). This study provided the first and nationwide prevalence data of LindaV infections in the domestic pig population in Austria and confirmed the already assumed low seroprevalence. An interesting finding is the substantial difference between the prevalence of LindaV (0.15 % at the individual level and 0.75 % at farm level) and the prevalence of APPV in the pig population. Prevalence data about APPV exist from countries worldwide demonstrating high genome prevalences of approximately 10 % to 20 % and seroprevalences of ≥ 60 % (Beer et al., 2017; Kaufmann et al., 2019; Michelitsch et al., 2019; Muñoz-González et al., 2017; Postel et al., 2017a; Yuan et al., 2021). These results are puzzling, considering the poor *in vitro* replication efficiency of APPV compared to LindaV (Hause et al., 2015; Lamp et al., 2017; Schwarz et al., 2017). On the other hand, BungoV shows good propagation in cultured cells and did not spread to other farms in Australia or to other countries either (Kirkland et al., 2015; Richter et al., 2014). A possible explanation could be that all three LindaV affected farms are farrow-to-finish operations, as the BungoV affected farm complex in Australia was (Kirkland et al., 2015). Infected, but clinically inconspicuous animals were not sold to other pig holdings, preventing viral transmission to naïve pig herds. Another possible reason could be the geographical location of the farms. The farms are located in rather low-dense pig areas with no other pig farms in the direct neighborhood, preventing indirect, e.g. airborne, transmission between herds.

Fortunately, a pig farm in Carinthia with clinical symptoms similar to the LindaV index case was brought to our attention in January 2021. We identified high loads of LindaV genome (> 10^8 GE/g tissue) in clinical samples of CT-affected piglets and performed a thorough diagnostic work-up of the novel LindaV outbreak. The diagnostic work-up revealed important data on the biology of LindaV, which are substantially different to the outcomes of our acute infection experiment.

Similar to the index case, the introduction of LindaV in the pig herd had dramatic impacts on more than half of the litters in the farrowing barn with very high preweaning mortalities of 80–90 %. Studies about APPV infections also demonstrate an increase in preweaning mortality of up to 46 % (Sutton et al., 2019), which is considerably lower compared to LindaV. The identification of several highly viremic, seronegative six-week-old piglets is a strong indicator for the existence of persistently or chronically infected animals. In contrast to the acute infection experiment, 10- to 100-fold higher viral loads were observed in the blood of infected pigs. The absence of neutralizing antibodies in two animals strongly supports the hypothesis of PI animals. Typically, PI animals shed large amounts of virus via secretions and excretions and are therefore the main source for viral spread in a population (Houe, 1995; Moennig and Becher, 2018; Ribbens et al., 2004). The analysis of pooled fecal and saliva swab samples confirmed this high shedding (viral loads between 10^6 and 10^7 GE/ml) in the case of LindaV infected piglets. In contrast, LindaV genome was hardly detectable in feces, nasal and oral swabs of acutely infected animals. The results from the LindaV field case in Carinthia are in accordance with the outcome of an experimental study about fetal infections with BungoV (Finlaison and Kirkland, 2020a).

The individual analysis of the different blood fractions (serum, plasma, PBMCs) highlights the importance of PBMCs in the infection process. Six out of nine viremic animals showed 10-fold higher viral loads in PBMCs compared to serum and plasma. The fact that PBMCs are the major target for pestiviral infections and are therefore the sample of choice has already been described for the classical pestiviruses CSFV (Summerfield et al., 1998) and BVDV (Bielefeldt-Ohmann et al., 1987).

We identified two additional LindaV strains, LindaV strain Austria2 from 2016 and Austria3 from 2021, in frame of our studies. Both of the strains could be easily propagated in porcine cell lines and showed similar growth characteristics to the initially detected LindaV strain Austria1. Sequencing of the full genomes enabled a detailed genetic and phylogenetic analysis. The high sequence identity of > 98 % in most of the coding regions indicates a close genetic relationship between the strains. In contrast, APPV strains show a high genetic divergence of up to 20 %, not only between strains found in different parts of the world but also within single countries (Beer et al., 2017; Guo et al., 2020; Mósena et al., 2018; Postel et al., 2017a; Zhang et al., 2017). Possible reasons for this difference between LindaV and APPV could be the low prevalence and circulation of LindaV accompanied by a lower immune selection pressure on the virus. The question whether the most recently identified strain

Austria3 evolved directly from the strains Austria1 or Austria2, respectively, cannot be answered with certainty. Based on evolutionary rates described for BVDV, ranging between 5.9×10^{-4} and 9.3×10^{-3} substitutions per site per year (Chernick et al., 2014; Luzzago et al., 2012), both LindaV strains from 2015 and 2016, respectively, could be direct ancestors of the strain from 2021.
Based on the clinical picture from the field, it can be assumed that the virulence of the two novel strains Austria2 and Austria3 is similar to the initially detected strain Austria1 from 2015.

Our seroepidemiological study could not identify a possible virus reservoir in the domestic pig population of Austria. Future research work should include sero-surveillance studies in the wild boar population, with a special focus on the regions surrounding the affected pig farms (i.e. Southern Austria) and also the neighbouring country Slovenia (< 50 km distance between each of the affected farms and the Slovenian border). In the case of APPV, a global distribution in the wild boar population has been demonstrated (Cagatay et al., 2018; Choe et al., 2020; Colom-Cadena et al., 2018; Stenberg et al., 2021). The wide distribution suggests a possible introduction of APPV from wild boars to the domestic pig population.
Especially in the last years, several authors demonstrated that pestivirus infections are not at all restricted to cloven-hoofed animals. The discovery of novel pestiviruses in bats, rats, harbour porpoises and pangolins raises the question of a possible wildlife reservoir of LindaV beyond pigs or ruminants (Firth et al., 2014; Gao et al., 2020; Jo et al., 2019; Wu et al., 2012).

In summary, our study provides important first data about the virulence and epidemiology of the novel pestivirus LindaV. In addition to LindaV strain Austria1 from the index farm in Styria, the full-genomic sequences of two novel strains (LindaV strain Austria2 in Styria and LindaV strain Austria3 in Carinthia) were identified in frame of this work. We described the already suggested low seroprevalence of LindaV in the domestic pig population, but retrospectively identified a second LindaV infected farm. Furthermore, we have identified many characteristic features of a “typical pestivirus” in LindaV. On the one hand, we have observed a low virulence in acutely infected animals with subsequent seroconversion and high neutralizing antibody titers. On the other hand, we have identified the ability to cause chronic (or possibly persistent) infections after transplacental infection with the presence of seronegative, but highly viremic animals. Still, LindaV remains an enigmatic pestivirus. With its rapid replication and high-titer growth in cultured cells, it has the abilities for a fast spread in the pig population. But even more than five years after the first description, LindaV is not widely distributed. On the contrary,

APPV grows very poorly to low-moderate titers after several passages in cultured cells, but is highly prevalent in all parts of the world. The reasons for this contradictory need to be addressed in future studies.

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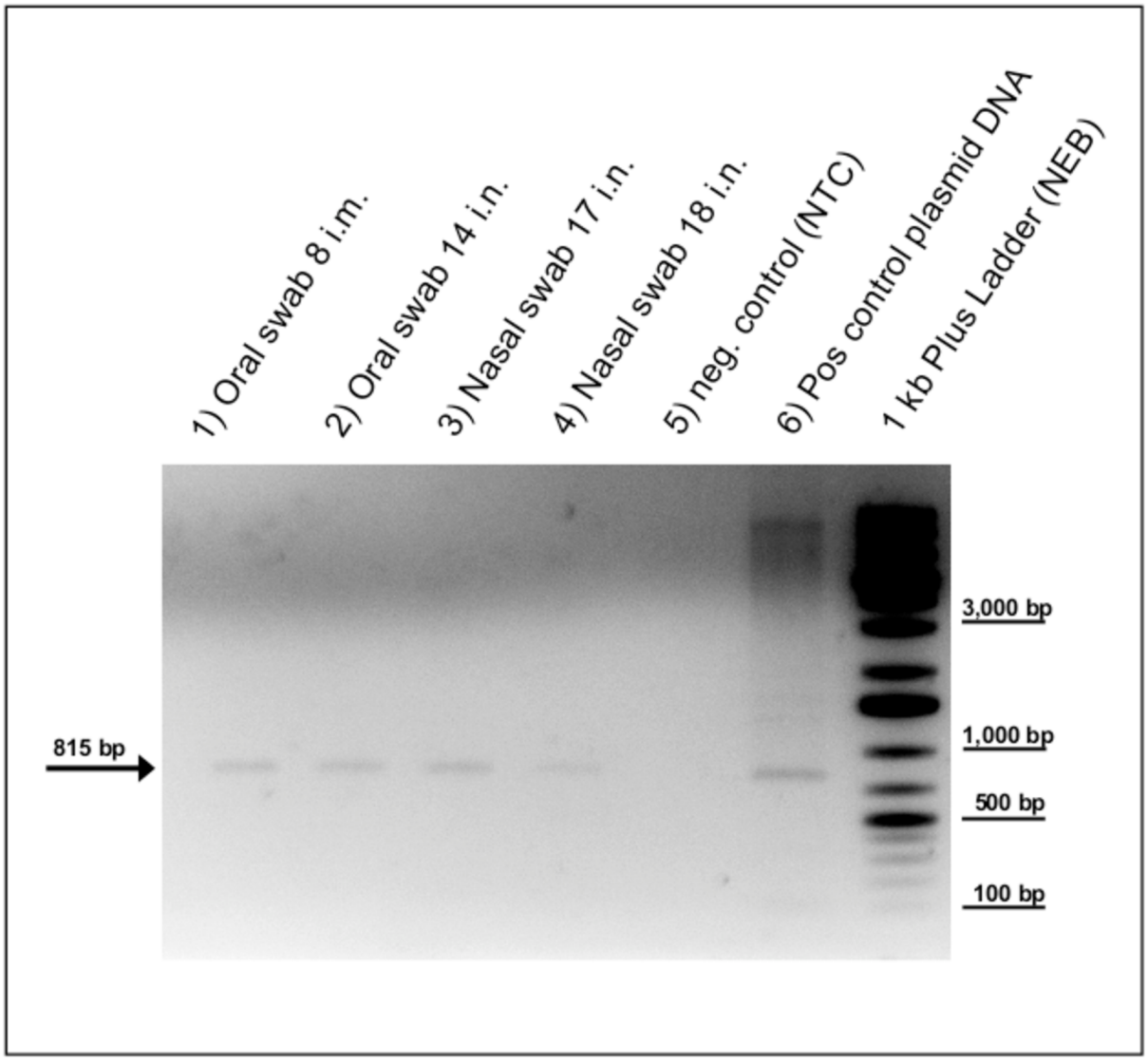
7. Appendix

7.1. Supplementary Material for Manuscript 1

Figure S1



7.2. Supplementary Material for Manuscript 2

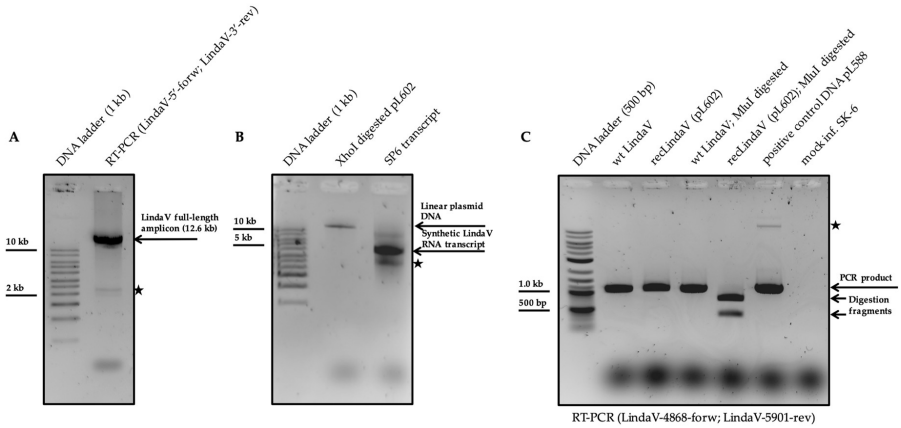


Figure S1. Establishment of reverse genetics for Linda virus (LindaV). (A) Genome length RT-PCR. Total RNA from a LindaV concentrate was transcribed using oligonucleotides hybridizing with the 5'- and 3'-ends of the LindaV genome including 5'-adapter sequences. The PCR products were subjected to agarose gel electrophoresis showing a specific LindaV genome amplicon as a strong double-stranded DNA band at 12.6 kb. Several smaller DNA bands of low intensity occur, of which two bands at about 2.0 kb labeled with asterisk. **(B)** Transcription of synthetic LindaV RNA. A molecular labeled plasmid clone of LindaV was constructed (pL602) by the integration of the LindaV DNA copy in a pBR322 backbone in between a SP6 promoter and a XhoI restriction endonuclease recognition site sequence. Plasmid DNA was digested with XhoI and transcribed using SP6 DNA dependent RNA polymerase. A strong signal of the single-stranded RNA molecule of 12.6 kb is visible after gel electrophoresis. The single-stranded RNA migrates more than two times faster than double-stranded DNA (please note the DNA marker). Weak signals of larger and smaller RNA molecules (labeled with asterisk) occur most likely due to incomplete plasmid DNA digestion and stop signal sequences for the SP6 polymerase, respectively. **(C)** Identification of the molecular marker. The infectious cDNA clone of LindaV was labeled by the integration of a synonymous MluI recognition site. A genome fragment, which includes this labeled sequence, was amplified by RT-PCR using the oligonucleotides LindaV-4868-forw and LindaV-5901-rev from cells infected with LindaV (wt LindaV) or with the molecular clone (recLindaV, pL602). After MluI digest of the PCR products, the specific DNA digestion fragments of 333 and 722 bp were only seen in cells infected with the molecular clone.

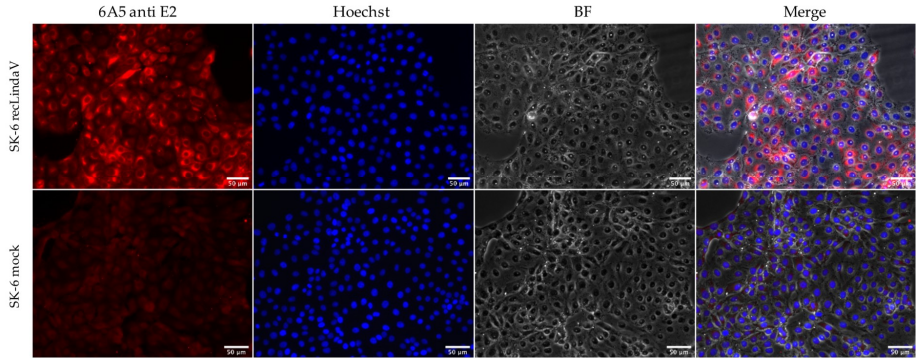


Figure S2. Monoclonal antibody (MAb) 6A5 detects the E2 expression in cells transfected with recombinant Linda virus (recLindaV) in an indirect immunofluorescence assay. SK-6 cells transfected with recLindaV were stained with the cross-reactive mouse MAb 6A5 (anti E2). Goat anti-mouse IgG conjugated with Cy3 was used as a secondary antibody. A LindaV E2 specific fluorescence is seen in cells transfected with LindaV RNA, while solely faint background signals occur in the non-transfected control cells. Cell nuclei were counterstained with Hoechst 33342. Images are shown at 20 x magnification. Scale bars represent 50 µm. BF, brightfield.

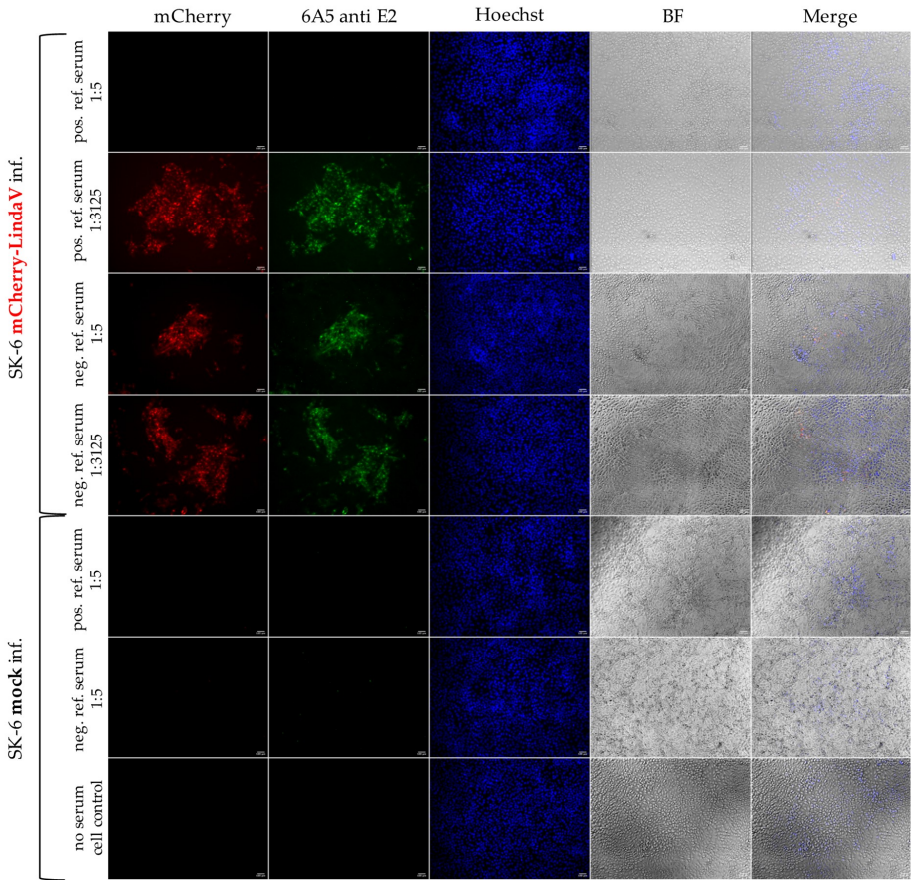


Figure S3. Serum virus neutralization assay (SVN assay) using an mCherry-Linda virus (mCherry-LindaV) and defined positive and negative reference Linda virus antisera. Five-fold serial dilutions of positive and negative antisera were prepared and incubated with an mCherry-LindaV dilution (100 $TCID_{50}$) for 2 h. SK-6 cells were added and incubated for 72-96 h. Cells were fixed and stained with the cross-reactive MAb 6A5 (anti E2). Goat anti-mouse IgG conjugated with FITC was used as a secondary antibody. Cell nuclei were counterstained with Hoechst 33342. Images are shown at 10 x magnification. Scale bars represent 100 µm. BF, brightfield.

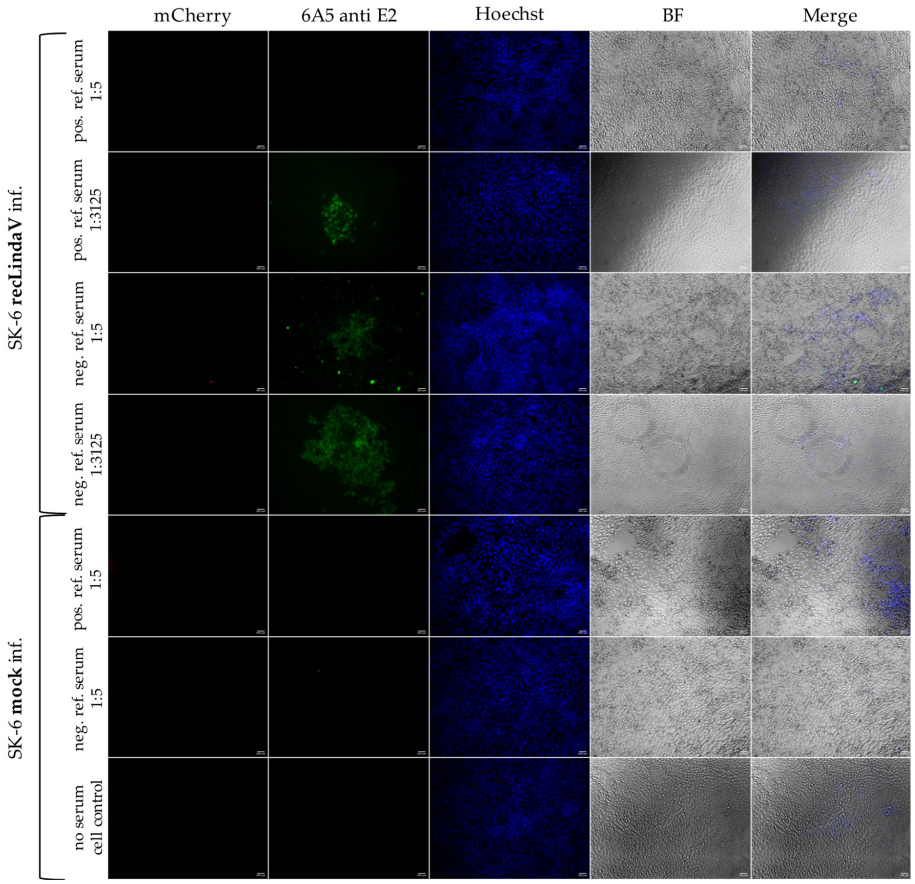


Figure S4. Serum virus neutralization (SVN) assay using a recombinant Linda virus (recLindaV) and defined positive and negative reference Linda virus antisera. Five-fold serial dilutions of positive and negative antisera were prepared and incubated with a recLindaV dilution (100 $TCID_{50}$) for 2 h. SK-6 cells were added and incubated for 72-96 h. Cells were fixed and stained with the cross-reactive MAb 6A5 (anti E2). Goat anti-mouse IgG conjugated with FITC was used as a secondary antibody. Cell nuclei were counterstained with Hoechst 33342. Note the absence of the red fluorescence signal (mCherry) compared to the images of the SVN assay with mCherry-LindaV depicted in Supplementary Figure 3. Images are shown at 10 x magnification. Scale bars represent 100 µm. BF, brightfield.

Table S1. Oligonucleotides used in this study.

Oligonucleotide name	Oligonucleotide sequence
LindaV-1-fw	5′-GTATAGCAGCAGTAGCTCAAGGCTG-3′
LindaV-544-rev	5′-GTGGTGGGTGCATGGGTCCGAAGC-3′
LindaV-446-fw	5′-ACATGTTCTGGCGGATGTACC-3′
LindaV-1496-rev	5′-TCTATATTGTACCAGTTACACCAGCC-3′
LindaV-1259-fw	5′-ACGACAAGAACGCAACAGATGTGC-3′
LindaV-2771-rev	5′-ATCAGTGTCTCAGTCGGATGGTC-3′
LindaV-2564-fw	5′-AGACTCAATGGTACCAAGCG-3′
LindaV-4024-rev	5′-ACGAAATAACTGTGAAGAGCATCGG-3′
LindaV-3796-fw	5′-ACATTGGTGTACGTAATAGGCATCG-3′
LindaV-5472-rev	5′-GCACTGCACCTTGGTTCTACCCATG-3′
LindaV-4868-fw	5′-CAGCAGACAGCAACAGTATACTATG-3′
LindaV-6821-rev	5′-CCATAACGTTGTGCCTGCAGCAG-3′
LindaV-6578-fw	5′-TGACATTACCAGACCTAGACAC-3′
LindaV-8103-rev	5′-TTCCATACCTGAGCTCACTGC-3′
LindaV-7873-fw	5′-TCGGCTCTGGCCAATTACAC-3′
LindaV-9258-rev	5′-CTCTCCAAGCTTGGTTTTGTG-3′
LindaV-9018-fw	5′-AGCACTGGTGAAGAGGTCATCC-3′
LindaV-10473-rev	5′-CTTGGACGTTTGAGCTCTGACG-3′
LindaV-10247-fw	5′-TAGAGAATCCTGGAGTGTGC-3′
LindaV-11777-rev	5′-TGTCCTATTACTTCCTTGTACGC-3′
LindaV-11559-fw	5′-GATGTGTACCAGGCTGGACTC-3′
LindaV-12607-rev	5′-GGGCCTCTTGGAACTGTAAGTAGTC-3′

7.3. Supplementary Material for Manuscript 3

Supplementary Table S1. Results of serum virus neutralization (SVN) assay (1/ND_{50}/mL), RT-qPCR assay (GE/mL; GE/g; GE/swab) and virus isolation from six-week-old nursery pigs. ND_{50}, 50% neutralization dose; GE, genome equivalents; PBMC, peripheral blood mononuclear cell.

	Animal ID	1/ND_{50}/mL	Serum GE/mL	Plasma GE/mL	PBMC GE/mL[1]	PBMC Virus Isolation	Feces GE/g	Saliva GE/swab
Six-week-old nursery pigs (P1-P15)	P1	1/434	4.46×10^5	1.04×10^6	2.58×10^5	+		
	P2	1/17.2	2.08×10^5	6.69×10^5	2.81×10^6	+		
	P3	neg.	2.62×10^7	2.03×10^7	3.16×10^8	+		
	P4	neg.	3.04×10^7	4.09×10^7	1.52×10^8	+		
	P5	1/10,640	neg.	neg.	neg.	-		
	P6	1/38.4	1.42×10^5	8.19×10^6	2.87×10^7	+		
	P7	1/968	2.26×10^5	4.80×10^4	9.61×10^4	-		
	P8	1/4,860	neg.	neg.	neg.	-		
	P9	1/434	neg.	neg.	neg.	-		
	P10	1/86.4	7.55×10^5	1.49×10^6	3.95×10^6	+		
	P11	1/38.4	8.15×10^6	1.63×10^7	1.66×10^8	+		
	P12	1/193.2	3.08×10^4	1.02×10^6	4.10×10^4	-		
	P13	1/2,180	neg.	neg.	neg.	-		
	P14	1/968	neg.	neg.	neg.	-		
	P15	1/10,640	neg.	neg.	neg.	-		
	P1-P15 (pooled sample)						2.65×10^6	1.17×10^6

[1] GE/mL PBMC pellet resuspended in 1 mL DMEM

Supplementary Table S2. Results of SVN assay (1/ND_{50}/mL), RT-qPCR assay (GE/mL, GE/g) and virus isolation from an eight-week-old euthanized nursery pig suffering from a paralysis of both hind legs. ND_{50}, 50% neutralization dose; GE, genome equivalents; PBMC, peripheral blood mononuclear cell.

Animal ID	1/ND_{50}/mL	Plasma GE/mL	PBMC GE/mL[1]	PBMC Virus Isolation	Tissue samples (GE/g; only positive results shown)				
					inguinal lymph node	medulla oblongata	tonsil	spinal ganglion	cerebellum
Euthanized nursery pig (eight-week-old)	1/10,640	neg.	neg.	-	2.28×10^6	2.25×10^5	2.56×10^6	5.36×10^6	1.19×10^6

[1] GE/mL PBMC pellet resuspended in 1 mL DMEM