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Seroprevalence of LINDA virus in the Austrian pig population between the years 2015 and 2020

Diploma thesis

University of Veterinary Medicine Vienna

submitted by

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1. List of abbreviations

APPV = Atypical porcine pestivirus

BungoV= Bungowannah virus

BVDV = Bovine viral diarrhea virus

C = Core Protein

CSV = Classical swine fever virus

ct = cycle threshold

ER = endoplasmic reticulum

IFN-1 = Interferon 1

LindaV = Linda virus

SNT = serum neutralization test

TGD= Tiergesundheitsdienst (Veterinary Health Service)

PI = Persistently infected animal

2. Introduction

2.1 Taxonomy

Linda Virus (LindaV) belongs to the genus *Pestivirus* within the family *Flaviviridae*, which consists of eleven approved species (Pestivirus A-K) and several unclassified species (International Committee on Taxonomy of Viruses. https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae/361/genus-pestivirus (Accessed: 14.06. 2021)). The classical pestiviruses are bovine viral diarrhea virus 1 (BVDV-1; *Pestivirus A*), bovine viral diarrhea virus 2 (BVDV-2; *Pestivirus B*), border disease virus (BDV; *Pestivirus D*) and classical swine fever virus (CSFV; *Pestivirus C*) (Smith et al., 2017). However, other pestivirus species have been discovered in pigs in the last few years, such as Bungowannah virus (BungoV; *Pestivirus F*), atypical porcine pestivirus (APPV; *Pestivirus K*) or the lateral-shaking inducing neuro-degenerative agent (Linda) virus (LindaV; tentatively *Pestivirus L*).

2.2 Pestiviral routes of transmission and infection

Pestiviruses infect pigs and ruminants, but they have also been detected in wild ruminants, wild boars (Nettleton, 1990; Leforban & Cariolet, 1992), rats (Firth et al., 2014) and bats (Wu et al., 2012). While direct transmission occurs by contact between animals, indirect transmission happens via contact with infectious secretions or contaminated feed (Laevens et al., 1999; Dewulf et al., 2002; Ribbens et al., 2004). Pestiviruses are excreted in all body secretions of an infected animal and transmission can occur horizontally or vertically by crossing the placenta and infecting the fetus (Moenning & Liess, 1995). The outcome of fetal infection is dependent on the time point of gestation and can range from resorption of the fetus to abortion, stillbirth and malformations or it leads to a persistent infection of the affected individuals (Moenning & Liess, 1995; Baker, 1987; Moenning & Plagemann, 1992). However, horizontal infection of immunocompetent animals often leads to healthy appearing individuals with no severe clinical signs, but a detectable seroconversion (Baule et al., 2001; Müller-Doblies et al., 2004; Kiesler et al., 2019).

That means as an example for APPV, one of the closest relatives which has been intensively investigated in the last years, that viral shedding occurs via feces and a high viral load of APPV was detected in the duodenum, pancreas, and colon (Postel et al., 2016). In addition, pestiviral

genome of APPV was found in salivary glands, therefore virus is also shed by saliva (Schwarz et al., 2017). Another transmission route of APPV is via infected semen, suggesting that artificial insemination may be an important route of transmission and therefore it must be controlled (Gatto et al., 2018).

Indirect transmission of APPV mainly occurs orally or nasopharingeally, but also infectious semen and iatrogenic diffusion, as well as environmental persistence are possible transmission ways (Lanyon et al., 2014; Niskanen & Lindberg, 2003; Iotti et al., 2019).

It is also interesting that in a study from Schwarz et al. (Schwarz et al., 2017) piglets with CT showed specific antibodies to APPV from birth up to eight weeks of age. The tremor symptoms disappeared completely until 14 weeks. However, viremia, antibody titer and virus shedding continued, viremia was markably reduced (Schwarz et al., 2017).

In a study of Cagatay et al. (Cagatay et al., 2019) vertically with APPV infected animals showed viremia from the first days after birth until slaughter. These piglets presented a high level of antibodies at six days of life, but antibodies were undetectable at 21 and/or 48 days of age. However horizontally infected animals showed viremia and high antibody titers up to 120 days post infection, suggesting the induction of protective immunity. For horizontally infected piglets the immune response was higher for the E2 protein, and E2 specific antibodies correlated with the presence of neutralizing antibodies (Cagatay et al., 2019; Dall Agnol et al., 2020). However, a correlation of neutralizing antibodies with E^{rns}-specific antibodies could not been found (Cagatay et al., 2018).

Because of the longevity of viral shedding, viremia, and the disappearance of specific antibodies against APPV, it can be hypothesized that a PI can be attributed to intrauterine-infected animals. These piglets can show a CT, which normally regresses over time (Munoz-Gonzalez et al., 2017; Schwarz et al., 2017; Postel et al., 2017). Horizontally infected animals develop a transient infection, through contact with PI animals, with a for several weeks' detectable viremia. However, these animals showed an active immune response against APPV over time whereby the virus became undetectable (Cagatay et al., 2019).

Another well explored example for pestiviral transmission and infection is BVDV. If viral infection occurs in the first days of gestation it will lead to resorption, while contact during the first third of pregnancy will probably result in an immunotolerant persistently infected progeny (PI). Infection during later stages of gestation can either result in malformations or in abortions.

Whereby PI animals are the main agent for disease distribution and persistence within a population (Ezanno et al., 2007; Iotti et al., 2019) and their excretion of BVDV is larger than that of an immunocompetent transiently infected animal, and lasts a lifetime (Lanyon et al., 2014). In addition, pregnant individuals carrying a PI progeny are latent persistently infected animals and hardly detectable by serological tests (Lanyon et al., 2014). As their offspring will become a PI, also these latent persistently infected animals are an important aspect of disease persistence. So that we can conclude that BVDV transmission between herds occurs generally via the direct movement of either a PI or a PI carrying individual (Lindberg & Alenius, 1999; Tinsley et al., 2012). If a PI is introduced to a new herd, transmission within the farm happens quite rapidly (within 1-8 months in the case of BVDV) (Cherry et al., 1998; Viet et al., 2004).

Based on the examples of APPV and BVDV we can conclude that dynamics of pestiviral infections can be assumed in two ways: persistently infected and transiently infected animals (Moenning, 2015; Ridpath, 2012).

2.3 Epidemiology and spread of novel pestiviruses in pigs

The first novel pestivirus which should be mentioned here is the Bungowannah virus, which is the closest relative of LindaV. BungoV was found on a farm in Australia in 2003 (Kirkland et al., 2007), but it was not discovered anywhere else in the world (Michelitsch et al., 2019; Mòsena et al., 2020; Abrahante et al., 2014). Further, APPV has first been reported from the US in 2015, and one years later from the Netherlands, whereby it was already widespread on both continents (Hause et al., 2015; De Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Michelitsch et al., 2019; Kaufmann et al., 2019). Shortly thereafter it was also discovered in Asian pig farms (Yuan et al., 2017).

In a recent study, serum samples deriving from German pigs, were analyzed for the presence of antibodies against APPV and BungoV (Michelitsch et al., 2019) in indirect immunofluorescence assays. While 16.3% of the analyzed sera had antibodies against APPV (detectable in 41.8% of the farms), no antibodies against BungoV were detected (Michelitsch et al., 2019).

In a recent study from Brazil serum samples from 320 backyard pig herds were examined for BungoV genome using RT-PCR assays. The results indicate that BungoV has not been

circulating in this backyard populations (Mòsena et al., 2020). Furthermore, in the US 64 serum samples, from undiagnosed cases of abortion or respiratory disease, were tested for BungoV genome using three specific qRT-PCR assays. Also, in this case BungoV could not be detected (Abrahante et al., 2014). Moreover, Kaufmann et al. searched for APPV in Switzerland using a RT-PCR assay resulting in a determined prevalence of 13% in slaughter pigs and 1% in breeding pigs, while no LindaV positive sample could be discovered (Kaufmann et al., 2019).

It is not surprising that LindaV has not been discovered anywhere else since its first discovery, like its closest relative BungoV which behaved the same way (Lamp et al., 2017). Studies about the epidemiological situation of LindaV are still rare and focused on the detection of LindaV genome (Kaufmann et al., 2019; Cagatay et al., 2018).
A recent study showed a low-level viremia in weaned piglets after experimental infection with LindaV, but a strong humoral immune response (Kiesler et al. 2019). Therefore, a serological method for the detection of LindaV seems more promising than direct detection methods.

2.4 Pestiviral genome organization

Pestiviruses are small, enveloped viruses with an RNA genome of 12 to 13 kilobases (kb) (Simmonds et al., 2017). They all contain an internal ribosomal entry site (IRES) such as *Picornaviridae*, *Pegiviruses* and the members of the *Hepacivirus* genera. The IRES contains determinants of viral growth, pathogenesis and cell type specificity and can be exchanged between viruses via a horizontal gene transfer (Arhab et al., 2020).
The pestiviral genome codes for one polyprotein, which is processed into four viral structural and eight non-structural proteins (Figure 1) via cellular and viral proteases (Rümenapf et al., 1993). The resulting pestiviral proteins are the structural glycoproteins, named E^{rns}, E1 and E2, a nucleocapsid protein termed Core, and the non-structural proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B (Smith et al.2017).

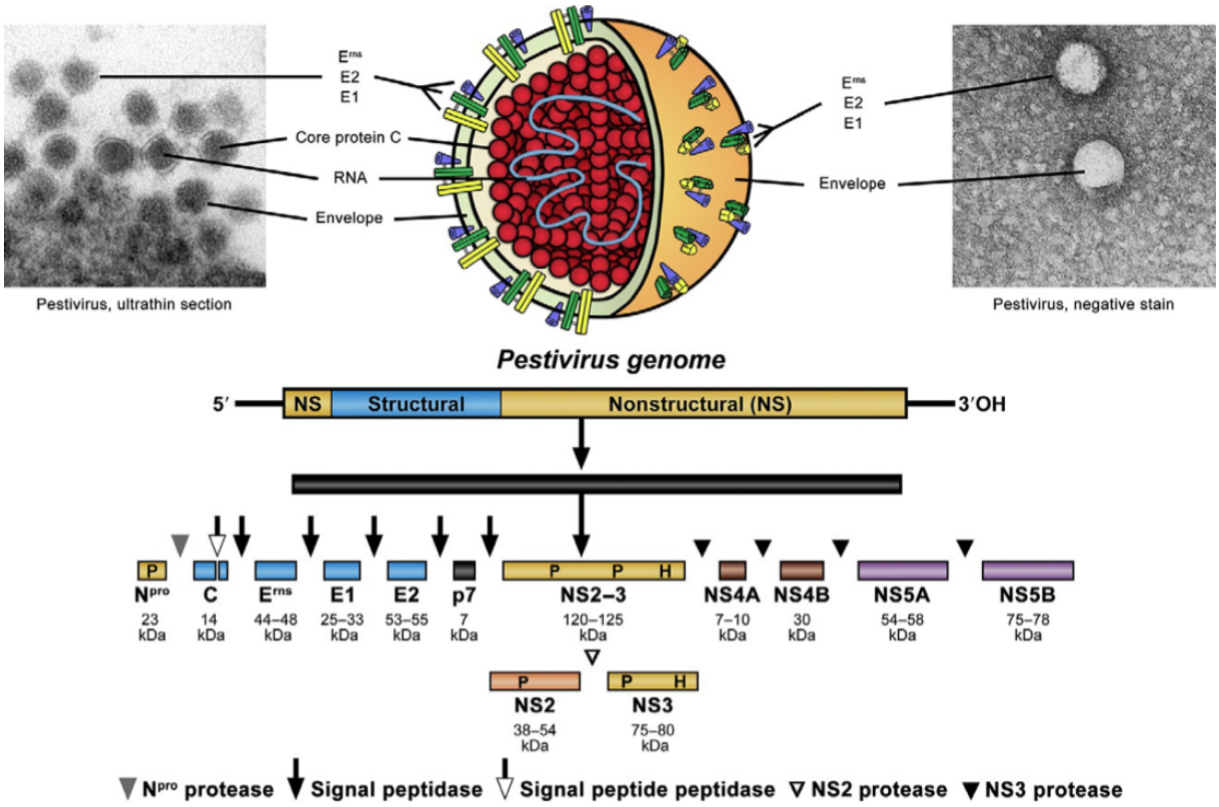

Figure 1:Pestiviral genome shown in a diagram with 2 electron microscopic pictures (BVDV on the left and CSFV on the right). The single long ORF, which encodes the pestiviral polyprotein, is processed by the proteases at the bottom. Processing leads to the listed proteins (Tautz et al., 2015).

N^{pro} is the first protein in the pestiviral genome and has a length of 168 amino acids (Tautz et al., 2015). It was discovered that N^{pro} can block interferon 1 (IFN-1) response to virus infection by reducing the intracellular level of interferon regulatory factor 3 (IRF-3) through its proteolytic activity (Bauhofer et al., 2007; Cao et al., 2019). Core Protein C is the first structural protein in the pestiviral polyprotein (Thiel et al., 1991) and binds RNA without specificity and with low affinity (Ivanyi-Nagi et al., 2008; Murray et al., 2008) and it is not essential for virus assembly, but for its virulence (Riedel et al., 2012). The Core protein merges into E^{rns} in the pestiviral polyprotein. E^{rns} is the first structural glycoprotein of the pestiviral genome, with an RNase activity and a membrane anchor ability (Tautz et al., 2015) and is followed by E1, which is the only pestiviral envelope protein which is rather unexplored, meaning that no structure or function is known yet (Tautz et al., 2015). The E2 domain represents the receptor-binding protein of pestiviruses and is consequently the main target for neutralizing antibodies (Deregt & Loewen, 1995; Weiland et al., 1990). This

includes that E2 alone is capable of inducing protection in animals and can be used as a vaccine (Bruschke et al., 1997; Zhang et al., 2018). In addition, E2 is the main reason for species diversity of pestiviruses (Liang et al., 2003; Miroslaw & Polak, 2020). The next protein p7 is a nonstructural protein required for the forming of infectious viral progeny and it is characterized as a hydrophobic, small integral membrane protein (Elbers et al., 1996; Wozniak et al., 2010; Zhao et al., 2017). Subsequently NS2 contains a cytoplasmatic domain, which includes a suspected Zn^{2+}-binding site (de Moerlooze et al., 1990) that is essential for an autoprotease domain located within NS2 (Lackner et al., 2004; Lackner et al., 2006; Dubrau et al., 2019). Then it comes to NS3 for which an original function in virus packing can be assumed (Tautz et al., 2015) followed by NS4A and NS4B. NS4A is a cofactor for the NS3 protease, but additionally it is also important for virus morphogenesis (Moulin et al., 2007; Tautz et al., 2000) and NS4B is essential for pestiviral replication, even though the function of NS4B has not been clarified yet (Grassmann et al., 2001; Weiskircher et al., 2009). The following protein NS5A is crucial for virus replication (Tellinghuisen et al., 2006) and it is able to regulate viral replication (Chen et al., 2012). NS5B is the last protein in the pestiviral genome and includes sequences specific for an RNA-dependent RNA polymerase (RdRp) (Steffens et al., 1999; Zhou et al., 2018).

2.5 LindaV outbreak in 2015 and following investigations

In 2015 a piglet producing farm located in the south of Austria reported of piglet losses during an episode of congenital tremor with a CT prevalence of 20-100% in affected litters (Lamp et al. 2017). Pestiviral antigen was detected in tissue samples of the CNS and also in the epithelium of the kidneys of diseased piglets in immunohistochemical analysis (Lamp et al. 2017) and the virus was successfully isolated. Subsequently the full genome of LindaV was determined with a length of 12,614 nt (Lamp et al. 2017). The genome of LindaV shows the highest similarity (of around 70%) to BungoV.

In 2017, a controlled animal infection experiment was conducted, where post-weaning piglets were infected with LindaV to detect the clinical outcome of infection in the immunocompetent host. Clinical signs were rare, but LindaV was detectable in the spleen and lymphatic organs and the infected individuals showed a high titer of neutralizing antibodies (Kiesler et al., 2019).

Even BungoV led only to a few clinical signs in an infection study with weaner piglets (Finlaison et al., 2012).

It is characteristic for pestiviruses that mononuclear cells and lymphoid organs are the first target of infection. Moreover, most pestiviruses are host-specific with mild clinical signs and restricted virus replication (Lanyon et al. 2014; Kiesler et al., 2019; Finlaison et al., 2012).

2.6 Aim of the study

The aim of this study was to determine the prevalence of LindaV infections in the domestic pig population of Austria. So far, LindaV has not been found anywhere else since its first description in 2015. Therefore, we hypothesize that LindaV is a rare pathogen and will be of low prevalence in Austrian pig herds. To assess this hypothesis the following methods will be used:

- Screening of 637 serum samples from sows and gilts (from five federal states of Austria, collected between the years 2015 and 2020) in a SNT using a fluorophorelabelled infectious cDNA clone of LindaV for high-throughput analysis
- Analysis of the serum samples in pools of five in a LindaV-specific RT-qPCR assay for the presence of LindaV RNA

3. Material and methods

3.1 Material

3.1.1 Serum samples

For LindaV-screening we analyzed 637 serum samples, obtained from 132 farms, from the years 2015-2020 from five federal states of Austria. 335 serum samples were collected in Upper Austria, 67 in Lower Austria, 214 in Styria, 11 in Carinthia and 10 in Burgenland. The sera were collected during herd health monitoring visits and archived at the University Clinic for Swine of the University of Veterinary Medicine in Vienna (302 serum samples) and at the veterinary health service in Upper Austria (TGD; 335 serum samples from the year 2020). In addition, 57 archived serum samples from the years 2016, 2019 and 2021 were obtained after the identification of a LindaV positive farm in Styria (farm O) during the screening. Overall, that results in a total number of 694 serum samples. The sampling years with the respective number of samples taken is listed in Figure 2:

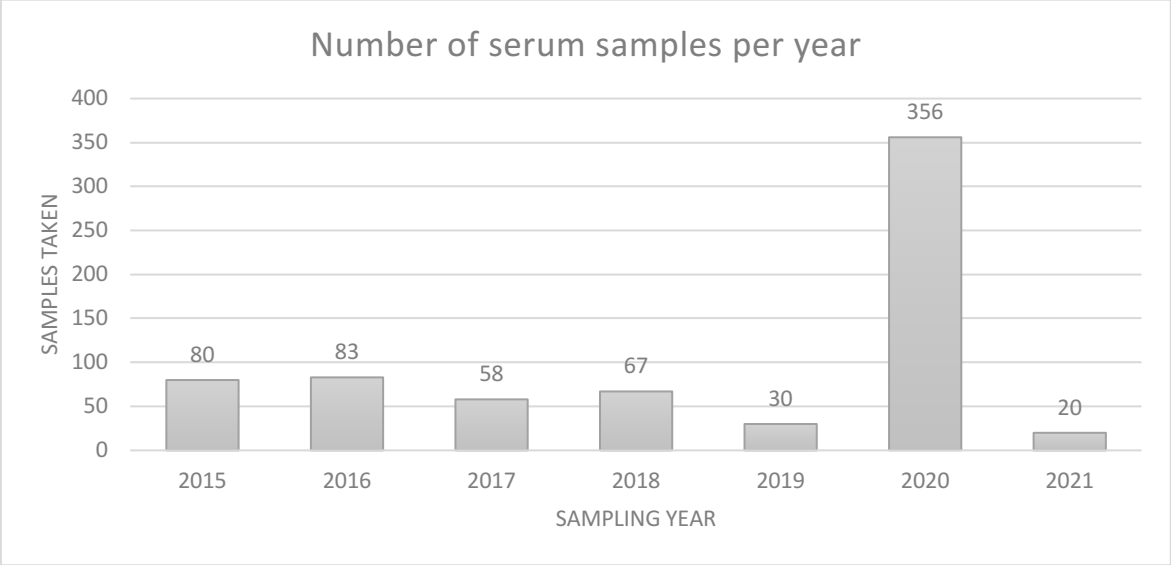

Figure 2: Sampling year with the respective amount of serum samples taken and archived at the University Clinic for Swine and the veterinary health service of Upper Austria. Figure created using Microsoft Excel.

Serum samples derived mainly from sows and gilts, as it is more likely to detect neutralizing antibodies against LindaV in these age groups. The number and origin of the serum samples is depicted in Figure 3:

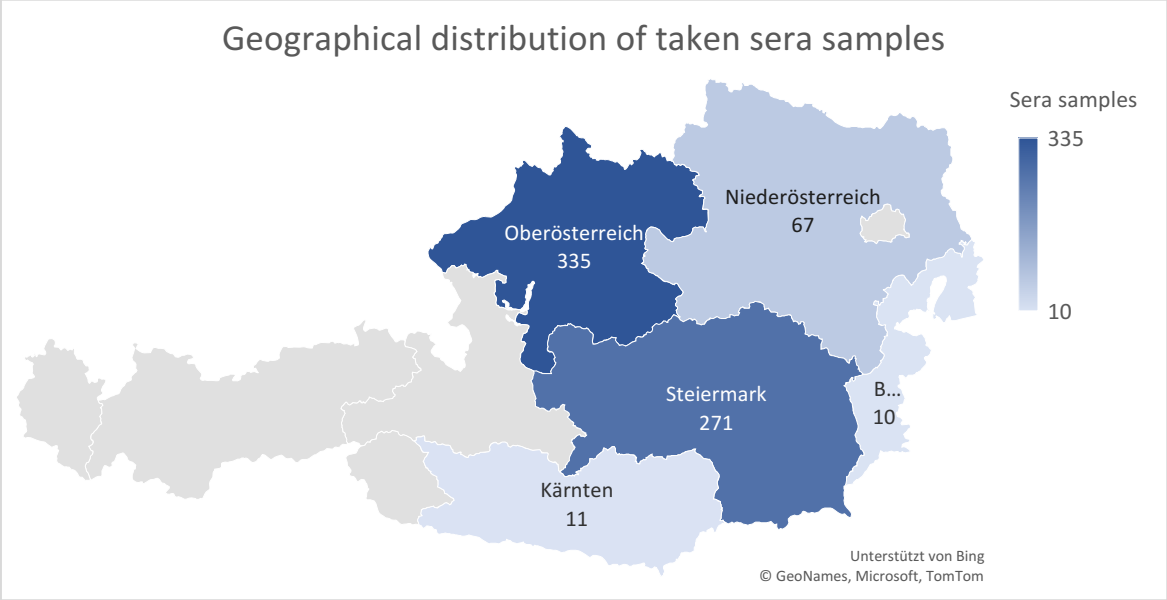

Figure 3:Geographical distribution of analyzed serum samples. Translation of Austrian federal states: Oberösterreich = Upper Austria; Niederösterreich = Lower Austria; Burgenland = Burgenland; Steiermark = Styria; Kärnten = Carinthia. Figure created using Microsoft Excel.

3.1.2 Reagents

2x Phanta Max Master Mix for PCR and Nested-PCR	Vazyme Biotech, China

3.1.3 Solutions, media, buffer, cells

DMEM (Dulbecco's Modified Eagle Medium) +10% FCS+1x P/S as cell culture medium	Biowest, France
DMEM for SK-6 cell culture	Biowest, France
Trypsin for SK-6 cell culture	GE Healthcare, UK
1kbp DNA ladder for agarose gel electrophoresis	Carl Roth GmbH & Co. KG, Germany

Agarose gel (0,8%) for gel electrophoresis	VWR Life Science, USA
DNA loading dye for agarose gel electrophoresis: 0,025% bromophenol blue; 0,2% orange G; 40% sucrose; 10mM Tris	New England Biolabs GmbH, Germany
SK-6 cells for SNT (Kasza et al., 1972)	Cell culture of the institute, Austria
Tris-acetate-EDTA (TAE) electrophoresis buffer: 2M Tris base; 1M acetic acid; 5mM EDTA, sterile filtrated; pH 8.0	
H_2O dd for cDNA and DNA purification (elution); PCR; Nested-PCR	
Paraformaldehyde (4% PFA) for cell fixation	
Phosphate buffered saline (PBS) for SNT	
PBS Tween 20 for SNT	
RNase free H_2O dd for cDNA synthesis	

3.1.4 Primers

LindaV E2 nested PCR:

- CST1067: Primer outer forward (nt 2489-2509)
  TTGAATGCAACTTCGAACTGC

- CST1068: Primer outer reverse (nt 3582-3605)
  ACTTGGTAGTGATTTAGCAACTCG

  - CST1069: Primer inner forward (nt 2564-2583)
    AGACTCAATGGTACCAAGCG

  - CST1070: Primer inner reverse (nt 3480-3500)
    ATCACGTGATCTTTTGCTGTC

LindaV RT-qPCR:

- CST756: LindaV TaqManPrimer forw. 200
  CACTGGWAAGGATCACCCACT

- CST757: LindaV TaqMan Probe 320
  Fam-ATAGGATGCCGGCGGATGCCCTGT-TamRa

- CST758: LindaV TaqManPrimer rev. 360
  AATYACAACGGATAWTMTTTATACTGG

3.1.5 Viruses

pAK24 - mCherry-labeled LindaV stock (1,78*10^5 $TCID_{50}$/ml), derived from in vitro transcribed RNA electroporated in SK-6 cells.

3.1.5.1 pAK24

pAK24 (Figure 4) is a plasmid containing the vector pBR322 as well as an SP6 promoter and the LindaV genome with the coding sequence of the fluorescent protein mCherry, inserted as

a fusion protein to the N terminus of the glycoprotein E2. This gives us the possibility to directly analyze the SNTs using a fluorescence microscope without immunofluorescence staining of the cells.

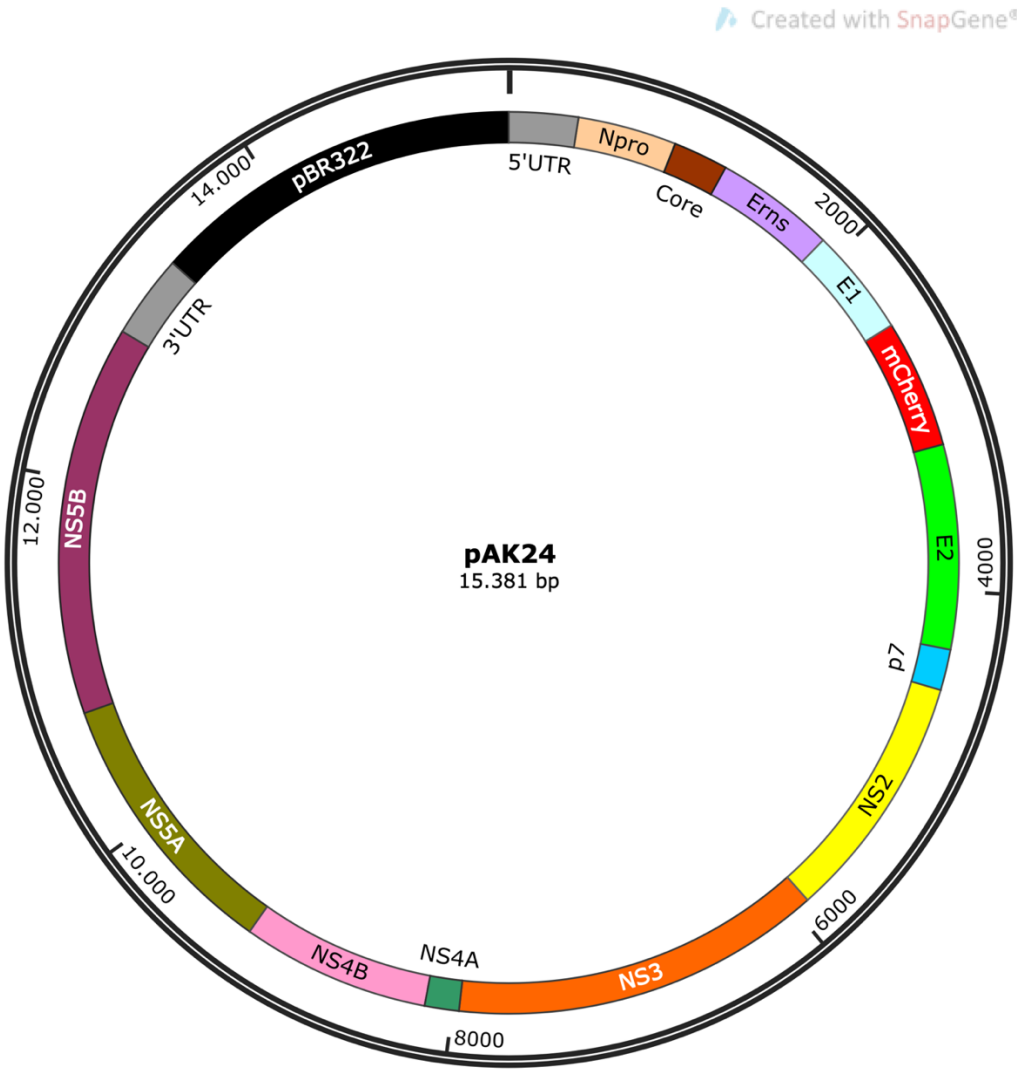

Figure 4: pAK24 with the location of the coding sequences of the LindaV proteins, as well as the inserted mCherry and the vector backbone pBR322. The coding sequences are flanked by a 5' untranslated region (UTR) and a 3'UTR. (5'UTR= 0-381nt); (Npro=382-927nt); (Core= 928-1230nt); (Erns= 1231-1893nt); (E1=1894-2487nt); (mCherry= 2488-3195nt); (E2= 3196-4320nt); (p7= 4321-4545nt); (NS2= 4546-5913nt); (NS3= 5914-7962nt); (NS4A= 7963-8151nt); (NS4B= 8152-9192nt); (NS5A= 9193-10704nt); (NS5B= 10705-12855nt); (3'UTR= 12856-13316nt); (pBR322= 13317-15381nt); Created by using the SnapGene Viewer [URL].

3.1.6 Laboratory equipment

Rotor Gene Q cycler	QIAGEN, Netherlands
FastGene Cycler	Nippon Genetics Europe GmbH, Germany
Eppendorf Thermomixer 5436	Eppendorf, Germany
UNIMAG ZX^3 Vortex mixer	UniEquip, Germany
Mini centrifuge	Nippon Genetics Europe GmbH, Germany
Mupid®- One electrophoresis system	Advance, Japan
Heraues® Biofuge® Pico microlitre centrifuge	Heraeus, Germany
Laminar Flow Biosafe 5-130	EHRET GmbH & Co KG, Austria
Vacuum pump ECOM-P 4153	Eppendorf, Germany
Manual single-channel pipettes Eppendorf research® plus 0,5-10 µl; 10-100 µl; 100-1000 µl	Eppendorf, Germany
Multipette® plus	Eppendorf, Germany
Eppendorf Xplorer® plus	Eppendorf, Germany
Incubator	Heraeus Instruments, Germany

Microwell plate washer	Nunc, Denmark
Fluorescence microscope Olympus IX70	Olympus, Japan
UVP Imager Biospectrum-AC with cool	Ultra-Violet Products Ltd., UK

3.1.7 Laboratory consumables

Pipette tips	Sarstedt AG & Co. KG, Germany
Microwell plates (96 wells)	Sarstedt AG & Co. KG, Germany
Graduated glass pipettes (5ml; 10ml; 25ml)	VWR Life Science Competence Center Erlangen, Germany
PCR tubes	STARLAB International GmbH, Germany
Crushed ice	

3.1.8 Kits

NEB Monarch PCR Purification Kit for cDNA and DNA purification	New England Biolabs GmbH, Germany
HiScript II 1st Strand cDNA Synthesis Kit for cDNA synthesis	Vazyme Biotech, China
NEB Luna One-Step RT-qPCR Kit for RT-qPCR	New England Biolabs GmbH, Germany
QIAamp viral RNA Mini Kit for cDNA synthesis	QIAGEN, Germany

3.2 Methods

3.2.1 Serum neutralization test (SNT)

With the SNT we wanted to determine the virus neutralizing activity against LindaV in the porcine serum samples. Serum samples were heat inactivated for 30 minutes at 56°C to minimize potential cell toxic effects. 96-well cell culture plates were used for the preparation of the serum dilutions. Initially, sera were diluted 1:5 and 1:10 in DMEM and prepared in duplicate. A fluorescently labelled-LindaV stock (pAK24; $1{,}78x10^5$ $TCID_{50}$/ml) was diluted to a final titer of 100 $TCID_{50}$/50µl, added to the serum dilutions and incubated for 2 h at 37°C. Positive and negative reference sera, serum controls (in a 1:5 dilution), cell controls and virus back titration in a four-fold serial dilution to calculate the $TCID_{50}$ of the respective virus dilution were included in each SNT run. After the incubation time of two hours, we added $1x10^4$ SK-6 cells to each well. Cells together with the serum/virus mixture were incubated for three to four days (37°C; 5% CO_2 concentration) until a strong fluorescence signal was detectable in virus infected cells. Cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at 4°C. The plates were washed twice with PBS-Tween 20 and directly analyzed using a fluorescence microscope.

3.2.2 Fluorescence microscopy

The SNTs were examined for the presence of virus infected cells (visible as strong fluorescence signals in the cell cytoplasm), as well as an intact cell monolayer to exclude cell toxic effects, using a fluorescence microscope.

3.2.3 Working with RNA and DNA

3.2.3.1 RNA extraction

After performing the SNTs, we analyzed the serum samples in pools of five in a LindaVspecific RT-qPCR for the presence of LindaV RNA. Therefore, we pooled the serum samples in groups of 5 (total volume of 140 µl; 28 µl per serum sample) and extracted viral RNA using the QIAamp Viral RNA Mini Kit according to the manufacturer´s instructions.

3.2.3.2 RT-qPCR

Once the RNA extraction of pooled serum samples was done, we continued with the RT-qPCR screening for LindaV RNA. Therefore, a mastermix with the following components was prepared (for one sample;1x + 15% overhead):

-Luna RT-Reaction Mix (2x) ..... 8,28 µl
-Luna RT-Enzyme Mix (20x) ..... 0,83 µl
-CST756 (LindaV for, 100µM)..... 0,066 µl
-CST758 (LindaV rev, 100µM) ..... 0,066 µl
-CST757 (LindaV Probe, 100µM)..... 0,033 µl
-H2Odd (Millipore)..... 5,625 µl

A total volume of 13 µl mastermix and 1 µl template (extracted serum pools) was added to each PCR tube, and cycled under the following conditions:

50°C → 10 min	Reverse transcription (RT) step	
95°C → 1 min	Initial denaturation	
95° → 10 sec	Denaturation	} x40
55° → 30 sec	Annealing	
60° → 1 min	Elongation (30-60 sec/kb)	
72° → 5 min	Final elongation	
8° → ∞	Hold	

In addition, we prepared a second mastermix for the detection of the internal control beta-actin with the following components (1x + 15% overhead):

-Luna RT-Reaction Mix (2x) ..... 8,28 µl
-Luna RT-Enzyme Mix (20x) ..... 0,83 µl
-Beta-Actin F (100µM) ..... 0,066 µl
-Beta-Actin R (100µM)..... 0,066 µl
-Beta-Actin HEX-Probe (100µM) ..... 0,033 µl
-H2Odd (Millipore)..... 5,625 µl

13 µl mastermix and 1 µl template were added to a PCR tube and the beta-actin RT-qPCR was cycled under the same conditions as the LindaV RT-qPCR.
Sera of LindaV positive pools were extracted separately and again analyzed in a second RT-qPCR run.

3.2.3.3 cDNA synthesis and purification

In a first step, RNA was denatured. For this, the following components were mixed:

-oligonucleotide CST1068 (2 µM)..... 1 µl

-RNase free H_2O dd ..... 2 µl

-RNA template ..... 5 µl

The so obtained mixture (mixture Step 1) was incubated at 65°C for 5 minutes and then cooled on ice for 2 minutes. For the cDNA synthesis the following components were mixed:

-mixture Step 1 ..... 8 µl

-2xRT Mix..... 10 µl

-HiScript II Enzyme Mix ..... 2 µl

The mixture was incubated at 50°C for 45 minutes. The cDNA was purified using the NEB Monarch PCR Purification Kit according to the manufacturer's instructions.

3.2.3.4 Polymerase chain reaction (PCR)

With the generated cDNA we were able to perform a PCR for amplification of the E2 coding sequence of LindaV. The PCR reactions contained the following components:

-2x Phanta Max MM ..... 12,5 µl

-CST 1067 (10µM)..... 1 µl

-CST 1068 (10µM)..... 1 µl

-H_2O dd ..... 8,5 µl

Oligonucleotides were selected to amplify the E2 coding sequence of LindaV (about 1.1 kb length). 2 µl of the cDNA template were added to 23 µl of MM. Positive, negative and non-template controls were also included in each PCR run. The PCRs were performed in a thermocycler under the following conditions:

95°C	→ 2 min	Initial denaturation	
95°	→ 15 sec	Denaturation	} x30
58°	→ 15 sec	Annealing	
72°	→ 35 sec	Elongation (30-60 sec/kb)	
72°	→ 5 min	Final elongation	
8°	→ ∞	Hold	

3.2.3.5 Agarose gel electrophoresis of PCR products

2 µl of loading dye was added to 4 µl PCR product, applied on an 0,8% agarose gel and gel electrophoresis was performed at 100 Volt for 30 minutes. A DNA size marker (1 kb ladder) was included on each gel.

3.2.3.6 DNA Purification

Purification of the PCR products was performed using the NEB Monarch PCR Purification Kit according to the manufacturer's instructions.

3.2.3.7 DNA Sequencing

Sequencing of the purified PCR products was performed by Eurofins Genomics GmbH (Germany). The sequences were aligned and analyzed with the computer programs DNAStrider and SnapGene Viewer.

4. Results

We initially screened 637 serum samples for the presence of LindaV-specific neutralizing antibodies in porcine serum samples. Out of these, 335 serum samples came from Upper Austria, 67 from Lower Austria, 214 were taken in Styria, 11 in Carinthia and 10 in Burgenland. The samples derived from sows and gilts and were taken between the years 2015 and 2020. In addition, 57 archived serum samples from the years 2016, 2019 and 2021 were obtained after the identification of a LindaV positive farm in Styria (farm O) during the SNT screening. Overall, that results in a total number of 694 serum samples. In addition to this diploma thesis the results of the study have already been published by Kiesler et al. (Kiesler et al., 2021). For this reason, we refer here to the corresponding paper which is attached to the document and complements this thesis.

The SNT was performed as described in 3.2.1 and out of the 637 serum samples, a single serum from the year 2019 yielded a positive result. This serum was again analyzed in a fivefold serial dilution and showed a high neutralizing antibody titer of 1/625.

That results in a prevalence of LindaV of 0.15 % based on the number of serum samples screened and a prevalence of 0.75% based on the number of farms tested.

Subsequently, serum samples were analyzed in pools of five for the presence of LindaV RNA in a LindaV RT-qPCR assay. LindaV RNA was not detectable in any of the serum pools in the RT-qPCR assay.

Since we were able to detect neutralizing antibodies in a serum of a sow housed in a farm in Styria, we analyzed 57 additional archived samples of the respective farm O. Out of these, 30 samples were taken in 2016, 7 in 2019 and 20 in 2021. Interestingly, this farm is located only a few kilometers away from the farm where LindaV occurred the first time in 2015. Out of these 57 serum samples, several sera from the years 2016 and 2019 showed neutralizing activity in the SNT. Three samples from the year 2016 even showed a particularly strong LindaV-neutralizing activity. The titers of these samples are sorted by the sampling years and displayed in Figure 5 and Figure 6.

Additionally, analysis of the archived sera from the farm O in the LindaV RT-qPCR assay, revealed one positive serum sample (S641) with a Ct value of 23.

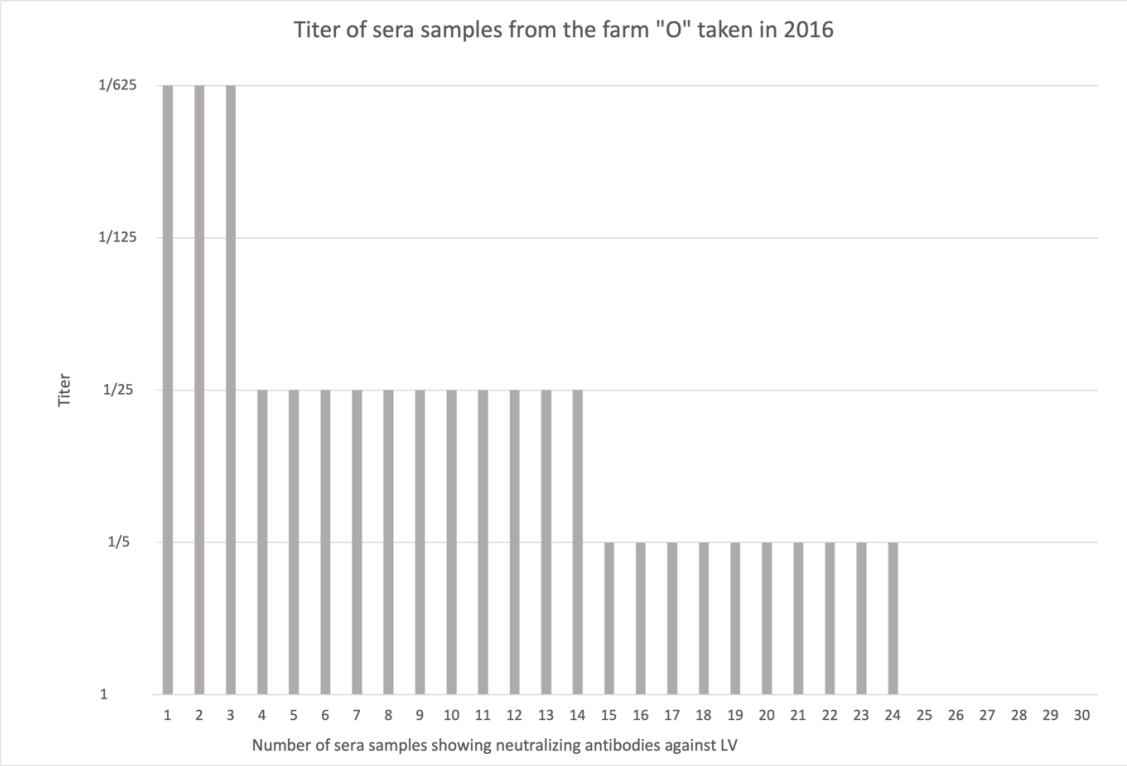

Figure 5: Number of serum samples from the farm O taken in 2016 and showing neutralizing antibodies against LindaV with the corresponding titer. Depiction created using Microsoft Excel.

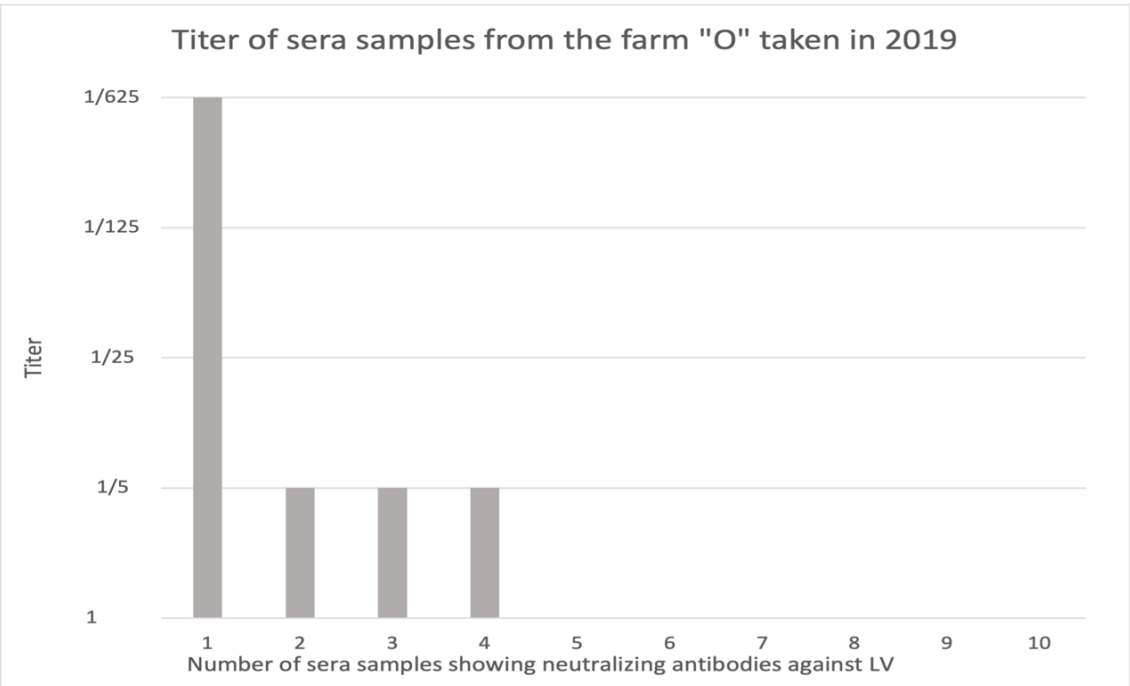

Figure 6: Number of serum samples from the farm O taken in 2019 and showing neutralizing antibodies against LindaV with the corresponding titer. Depiction created using Microsoft Excel.

As the LindaV RT-qPCR amplifies only a small fragment of about 180 bp in the 5'-UTR of the viral RNA, we analyzed serum sample S641 in a conventional two-step RT-PCR, amplifying the coding region of the glycoprotein E2. The PCR product was analyzed on an agarose gel (Figure 7) for the correct size and the nucleotide sequence was determined by Sanger sequencing.

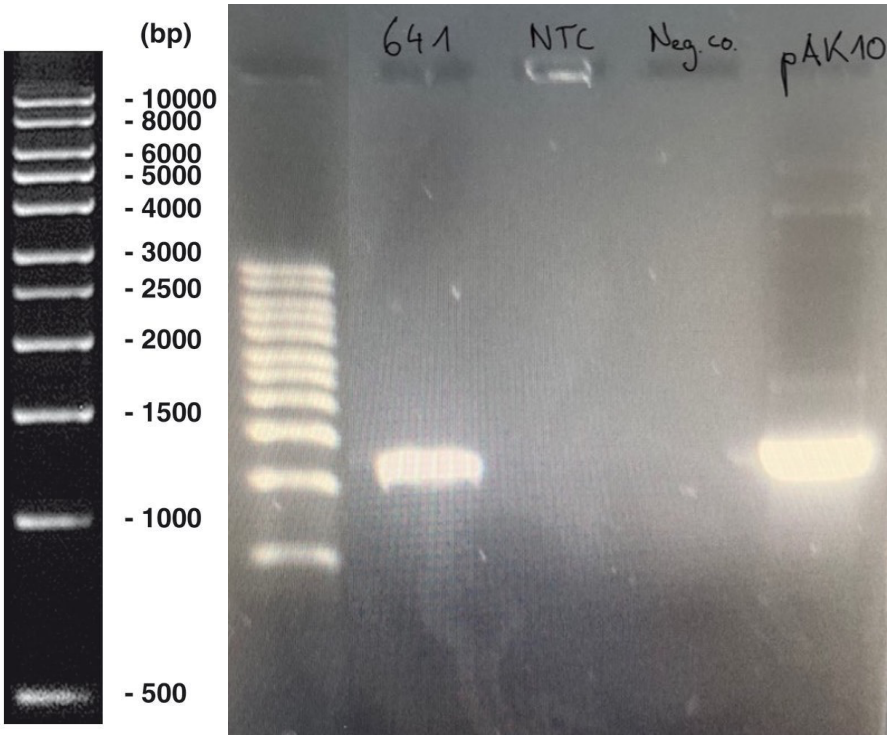

Figure 7: On the left: Fa. Roth 1kbp DNA ladder [URL] (Accessed: 09.03.2021); On the right: 1kbp DNA ladder, sample 641, non-template control (NTC), negative control (Neg.co) and pAK10 (plasmid containing the full-length genomic cDNA of LindaV) as positive control.

The PCR product of S641 showed a strong band at about 1.1 kb, analogous to the amplicon of the positive control. No bands were detectable in lanes of the non-template control (NTC) and the negative control. The PCR product of sample S641 was purified and Sanger sequencing was conducted.

We generated a consensus sequence by aligning the forward and reverse sequences and compared it with the respective E2 coding region of the originally found LindaV sequence using the DNAStrider software [URL]. This was done for the nucleotide sequence and the amino acid sequence and is displayed in the figures below (Figure 8; Figure 9).

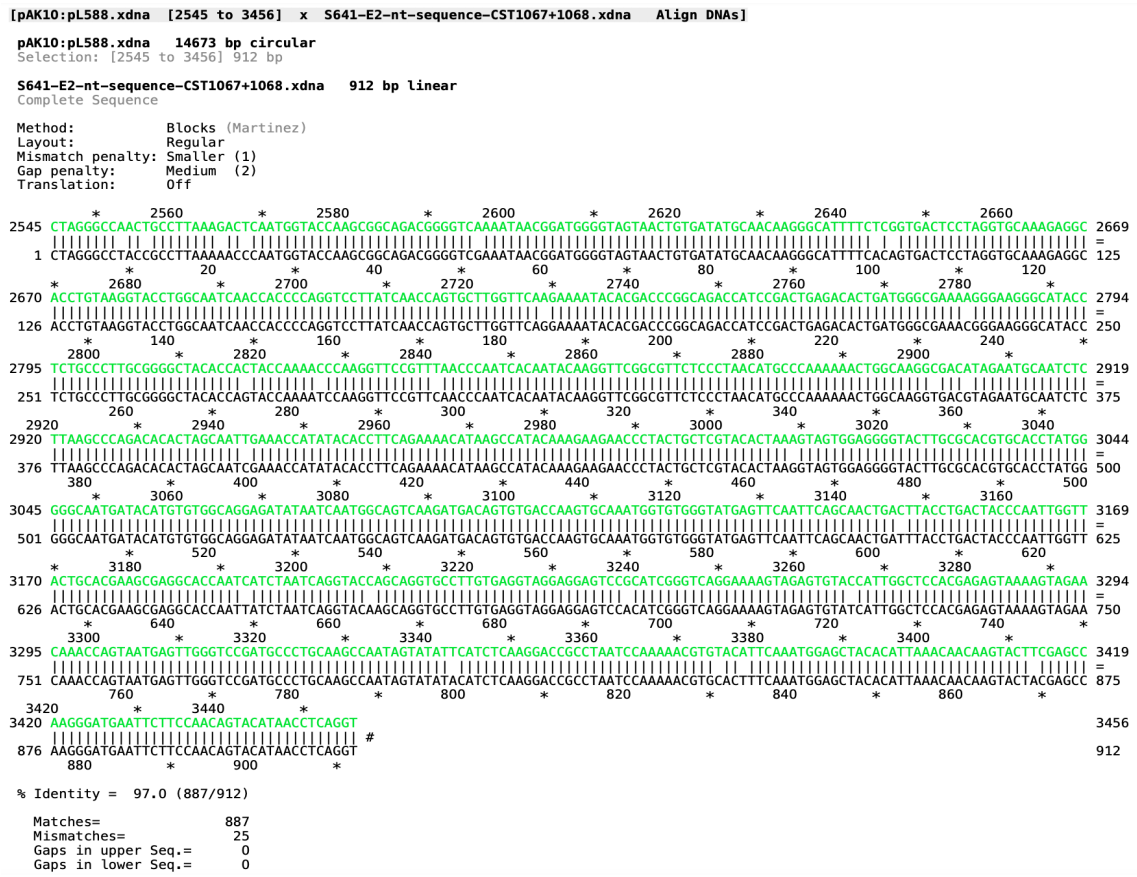

Figure 8: Alignment of the LindaV E2 coding region (green) with the respective sequence of sample S641 (black). Figure created using the DNAStrider [URL].

Figure 9: LindaV amino acid sequence (green) aligned with the E2 domain of sample S641 (at the bottom; black). The middle line shows the consensus sequence, with homologous amino acids displayed with "+" and mismatched amino acids marked with a gap. Figure created using the DNAStrider [URL].

In Figure 8 the nucleotide sequence of LindaV genome aligned with the corresponding E2 coding region of sample S641 is shown. Out of the 912 aligned nucleotides we found 887 matches and 25 mismatches. Consequently, a sequence identity of 97% was calculated. Aligning the amino acid sequences of LindaV and sample S641 (Figure 9) we got an identity of 95% with 291 matches and 13 mismatches. Out of the 13 mismatches 9 showed a homologous amino acid (2% homology) leading to a total identity of 98.7%.

5. Discussion

As mentioned in 2.6 the aim of this study was to determine the prevalence of LindaV infections in the domestic pig population of Austria. So far, studies about the LindaV prevalence have been rare and the virus has not been found anywhere else in the world, since its first description in 2015. For this reason, we conducted the first epidemiological screening of Austrian pig herds for LindaV infections. In total, 637 serum samples from the federal states with the highest pig density in Austria were provided by the University Clinic for Swine of the University of Veterinary Medicine in Vienna (302 serum samples) and by the veterinary health service in Upper Austria (TGD; 335 serum samples from the year 2020) and analyzed in a SNT.

The possibility of cross-neutralization between LindaV and other pestiviruses was excluded in a previous study (Kiesler et al. 2019). Therefore, the SNT provides the highest sensitivity and specificity compared to other serological detection methods (e.g., ELISA). The disadvantages of the SNT consist of its time-consuming preparation and read-out, which also limits its size number, and the necessity of a cell culture and sterile laboratory conditions. We overcame these limitations with the use of a fluorophore-labelled cDNA clone of LindaV, so we could directly analyze the assay without the need of an immunofluorescence staining. Due to the results of the SNT with the 637 serum samples we were able to calculate the prevalence of LindaV in Austria between the years of 2015-2020 to be 0.15% (based on the animal number) and 0.75% (based on farm level) with only a single sample showing LindaVneutralizing antibodies. The calculated, preliminary prevalence should be confirmed in larger epidemiological studies, since the outcome was limited by the sample size number, which was not representative for Austrian pig herds, but covered the main pig producing federal states of Austria.

Not surprisingly, the only antibody-positive sample derived from a farm (farm O) located only a few kilometers away from the farm where the first outbreak of LindaV occurred (Lamp et al., 2017). To gain deeper insights into the serostatus of the pig herd in farm O and a potential LindaV circulation, we analyzed 57 additional serum samples from this farm. We could identify several animals with variable titers of LindaV-neutralizing antibodies. Several serum samples from 2016 and a few sera from 2019 showed intermediate to high neutralizing antibody titers. Sera taken in 2021 were all negative in the SNT (Figure 5 and 6). From these findings we can speculate that LindaV was introduced in the farm in 2016, circulated within the pig herd for three years and disappeared from the farm after 2019. Nevertheless, a representative sample number throughout the years would be necessary to reconstruct the infection events in the farm.

If we compare the prevalence of LindaV to that of APPV, where a large number of epidemiological studies is available, the prevalence of LindaV seems to be very low. Hause et al. calculated a prevalence of APPV of 94% in the US indicating a more or less complete infection of the US pigs (Hause et al., 2015). In contrast a screening of 369 sera from healthy adult pigs in Germany showed the existence of APPV in Germany with an individual prevalence of 2.4% and 10% at farm level (Postel et al., 2016). A second study from Germany about the seroprevalence of APPV in German pig farms took place in 2019 and determined the seroprevalence of samples from 2009/2010 to be 15.3% and samples from 2018 with a seroprevalence of 17.5% (Michelitsch et al., 2019). Additionally, in Switzerland the prevalence of APPV has been calculated to be 13% in pigs for slaughter and less than 1% in breeding pigs in sera obtained between 1986 and 2018 (Kaufmann et al., 2019). A study from China calculated the prevalence of APPV to be 5.2% in the tested serum samples, only two years after its first description (Yuan et al., 2017). Therefore, we can assert that APPV was quickly recognized as pathogen with a worldwide importance after its first description in 2015. To the current knowledge, this does not apply to LindaV or its closest relative BungoV.

In addition to the SNT, we performed a LindaV RT-qPCR analysis of pooled serum samples in which all of the pooled serum samples were negative. In comparison to the results of the SNT, where we detected one antibody positive sample, we can conclude that a serological detection method is more suitable for conducting epidemiological studies of LindaV

infections. However, the analysis of additional 57 serum samples from farm O revealed a single genome-positive, but seronegative serum sample (sample 641), among several seropositive, genome-negative samples.

It is not surprising that the most important factor of survival strategies of pestiviruses is their ability to cause persistent infections in their hosts (Thiel et al., 1996). This animal (sample 641) could represent a persistently or chronically infected animal, which is the main source for transmission of pestiviruses as those animals shed large amounts of virus (Shoemaker et al., 2009; Smirnova et al., 2012; Smirnova et al., 2014; Chase, 2013). Infection with BVDV between day 40 and 120 of gestation can lead to the birth of persistently infected animals (Smirnova et al., 2012; Smirnova et al., 2014). Experimental studies investigating fetal LindaV infections and the critical stage of gestation have not been done so far.

The sample 641 was taken in 2016, the potential year of the introduction of LindaV in farm O, since most antibody positive serum samples were found in this year (Figure 5).

Finally, Sanger sequencing and sequence analysis of the E2 coding region of sample 641 was performed and a sequence identity of approximately 97% determined, so that we concluded that sample 641 forms a novel strain of LindaV. The mismatches are likely due to point mutations, since in all 25 cases only one nucleotide was exchanged. This high similarity to the

LindaV genome from 2015 could indicate a low immune selection pressure on the virus (Kiesler et al., 2021).

In conclusion the assumed low prevalence of LindaV infections (0.15%) was confirmed in Austrian pig herds. We were able to identify a pig farm (farm O) in the proximity to the original outbreak (farm L) in which the collected serum samples suggest, that LindaV circulated for at least 3 years. From a seronegative preserved sample of this farm sequences of a variant LindaV strain was identified showing an identity of 97% to the original LindaV sequence in the E2 coding region. This is reminiscent of the endemic character of BungoV the spread of which is confined to a single pig holding.

6. Summary

The aim of this study was to determine the prevalence of LindaV in the Austrian pig population. For this, 637 archived serum samples from 132 pig farms in five different federal states of Austria were analyzed. The porcine serum samples were collected between the years 2015 and 2020. The as "gold standard" established serum neutralization test (SNT) was used for the detection of neutralizing antibodies against LindaV. Only a single serum sample showed LindaV-neutralizing-antibodies (prevalence of 0.15%). Interestingly, this sample derived from a farm (farm O) a few kilometers away (10 km) from the location of the first virus detection. For further investigations in the respective farm, 57 additional samples from farm O were analyzed, with several sera showing intermediate to high neutralizing antibody titers. In addition, all serum samples were examined in pools for LindaV genome by RTqPCR. LindaV RNA was not detectable in any of the pooled sera, but a seronegative serum from farm O, showed a positive result in the RT-qPCR assay. This sample was further analyzed in a conventional RT-PCR, covering the E2 coding region, and the sequence was determined by Sanger sequencing. Sequence analysis revealed an identity of 97% (based on the nucleotide sequence) and 95% (based on the amino acid sequence) compared to the originally found LindaV sequence.
Thereby it was not only possible to determine the prevalence of LindaV between 2015-2020 (0.15%), but also to identify a novel LindaV strain.

7. Zusammenfassung

Ziel dieser Arbeit war es die Prävalenz des LindaV in der österreichischen Schweinepopulation zu bestimmen. Dafür wurden 637 archivierte Serumproben aus den Jahren 2015-2020 herangezogen, welche aus fünf unterschiedlichen österreichischen Bundesländern stammten. Als Nachweismethode wurde der als „Goldstandard" geltende Serumneutralisationstest (SNT) verwendet. Dabei zeigte eine der Proben LindaVneutralisierende Antikörper (Prävalenz von 0,15%). Interessanterweise stammte sie aus einem Betrieb (Betrieb O) nur wenige Kilometer entfernt vom Ort des ersten Virusnachweises. Zur weiteren Untersuchung dieses neuen LindaV-Stammes wurden zusätzliche 57 Proben vom Betrieb O herangezogen, wobei zahlreiche Seren LindaV-neutralisierende Antikörper zeigten.

Zusätzlich wurden alle Serumproben in 5er-Pools in einer LindaV RT-qPCR auf das Vorhandensein von LindaV Genom untersucht. Auf diese Weise fanden wir LindaV-RNA in einer der Proben vom Betrieb O, welche wir zur weiteren Charakterisierung im E2 codierenden Bereich sequenzierten. Die so erhaltene E2 Sequenz wurde mit jener des LindaV-Stammes des Erstausbruches verglichen und auf Mutationen untersucht. Dabei waren 25 Punktmutationen in einer 912nt Sequenz nachweisbar wodurch die Annahme eines neuen LindaV-Stammes bewiesen war. So konnte mit dieser Arbeit nicht nur die ursprünglich gesuchte Prävalenz von LindaV zwischen 2015-2020 bestimmt werden (0,15%), sondern auch der bisher einzige neue Stamm von LindaV nachgewiesen werden.

8. Literature

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9. List of figures

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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′-TAATAGGGGAGCCCAGGGTATGGTGAGCAAGGGCGAGGAG-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.

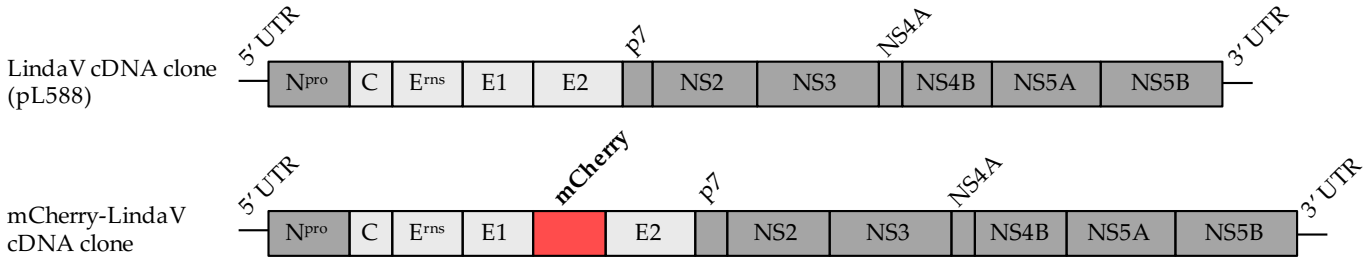

Figure 1. Schematic representation of the construction of an mCherry-labeled full genome infectious cDNA clone of Linda virus (LindaV). The coding sequence of the fluorescent protein mCherry was fused to the 5′-end of the LindaV E2 coding sequence in a LindaV cDNA backbone using a DNA assembly reaction. 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).

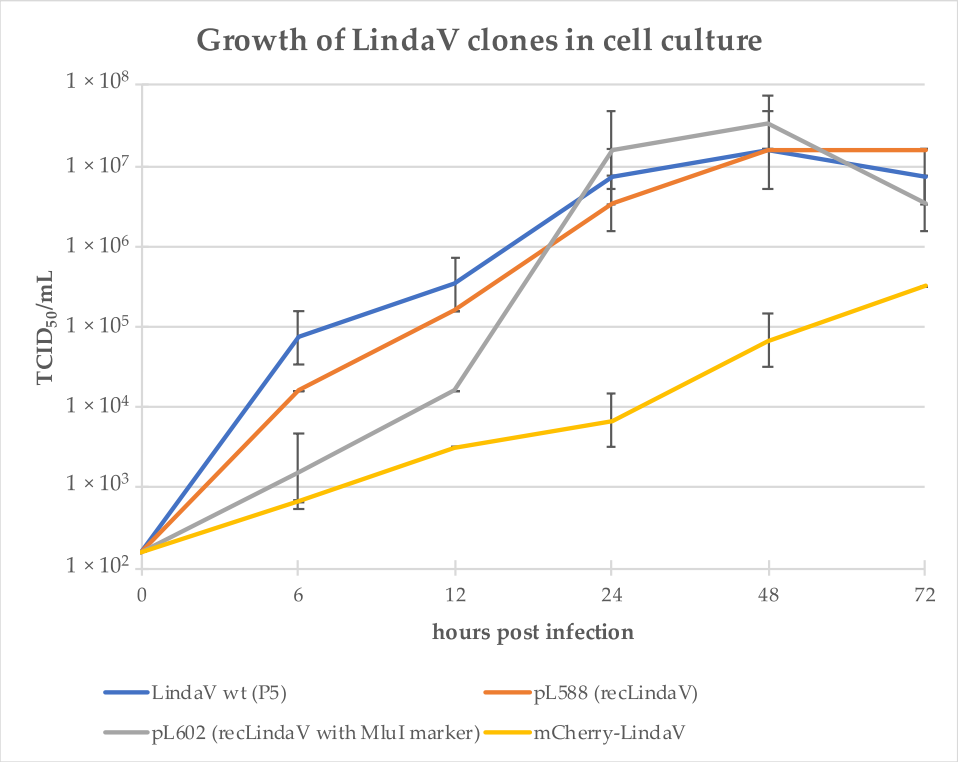

Figure 2. Cell culture growth of wild-type Linda virus (LindaV wt), recombinant Linda virus (recLindaV) and mCherry-Linda virus (mCherry-LindaV) clones. A monolayer of SK-6 cells was infected with 1×10^7 $TCID_{50}$ of the indicated viruses (MOI > 1). Two hours after infection, the cells were washed 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.

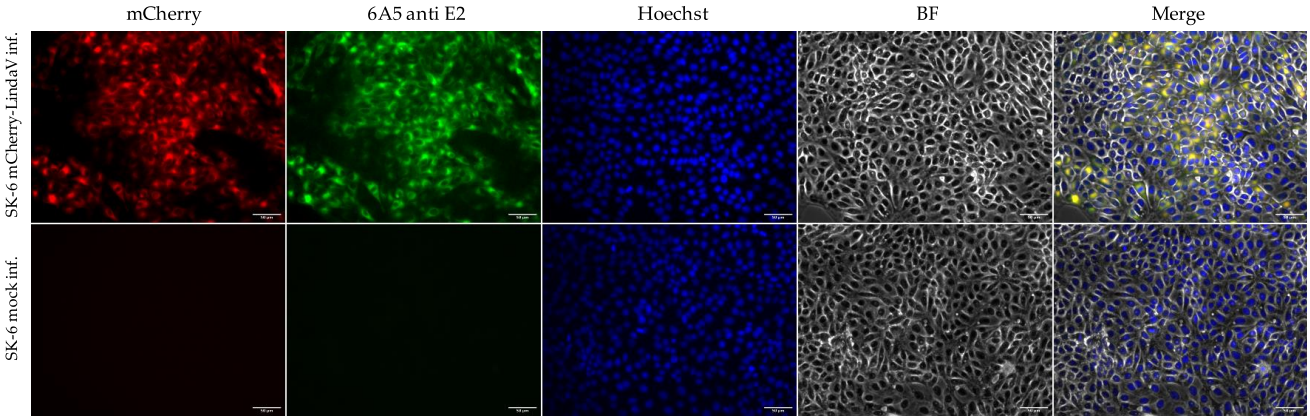

Figure 3. Indirect immunofluorescence assay of SK-6 cells infected with a fluorophore labeled Linda virus clone (mCherry-LindaV). SK-6 cells were infected with mCherry-LindaV and fixed at 48 h post infection. Cells were stained with the cross-reactive mouse MAb 6A5 (anti E2). Goat anti-mouse IgG conjugated 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.

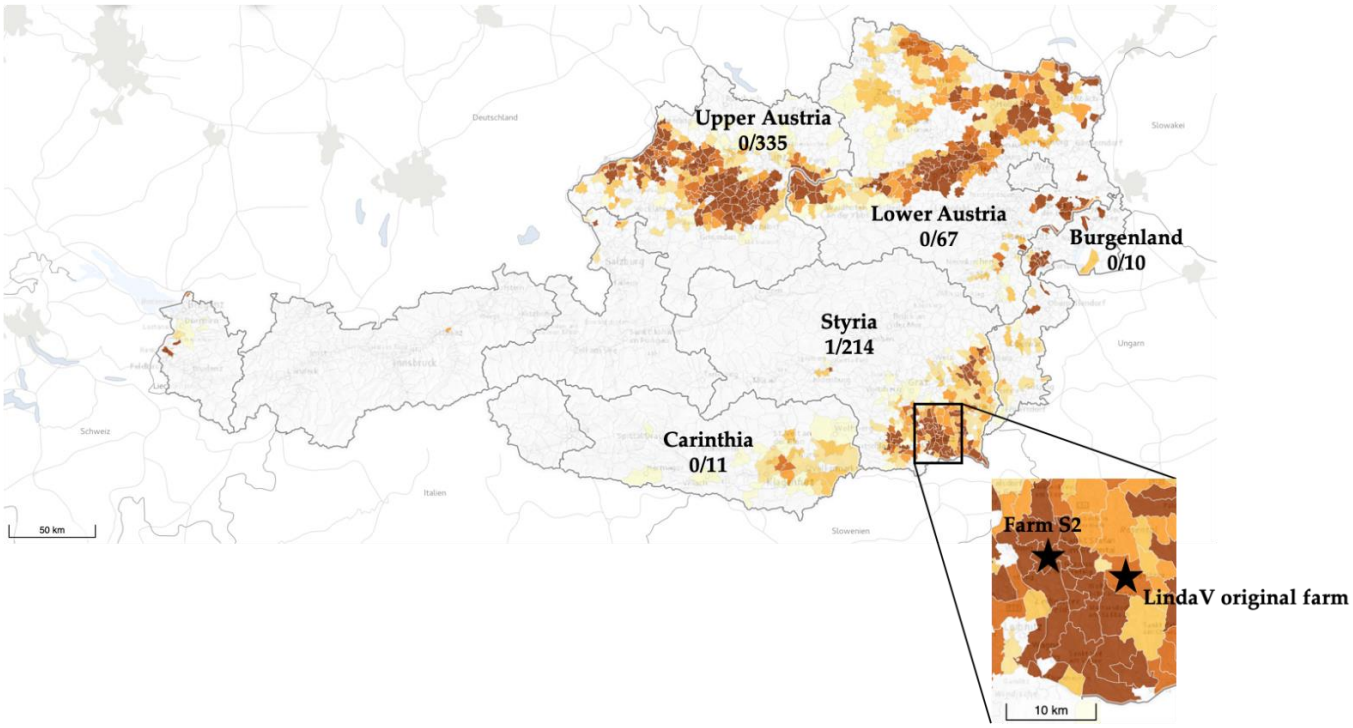

Figure 4. Presence of Linda virus (LindaV) neutralizing antibodies in porcine serum samples from Austrian pig farms. A total of 637 serum samples from five federal states of Austria (Upper Austria, Lower Austria, Styria, Carinthia and Burgenland) were analyzed in a LindaV SVN assay. The number of LindaV 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).

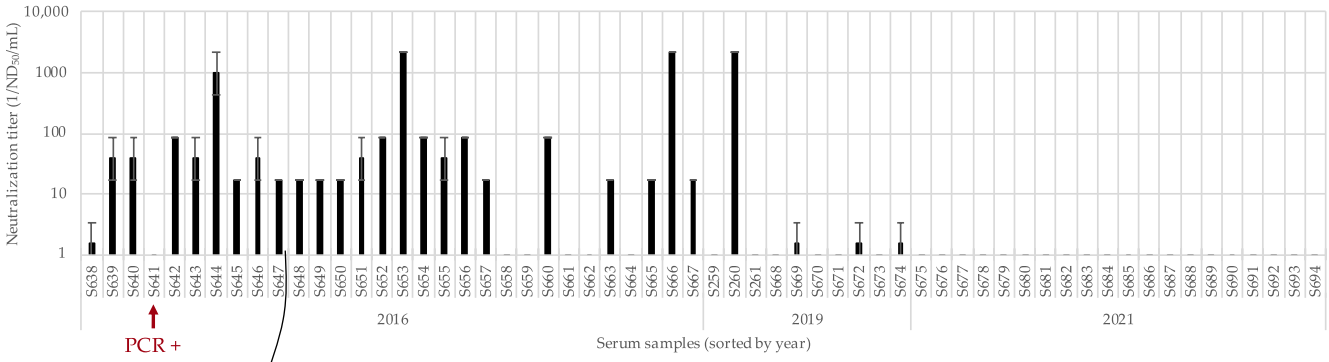

Figure 5. Virus neutralization titers of serum samples from Farm S2 from the years 2016, 2019 and 2021. Sera were analyzed in duplicate in a five-fold serial dilution starting at a dilution of 1/5 in an SVN assay. Neutralization titers (ND_{50}/mL) were calculated using the Spearman-Kaerber method. Neutralization 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.

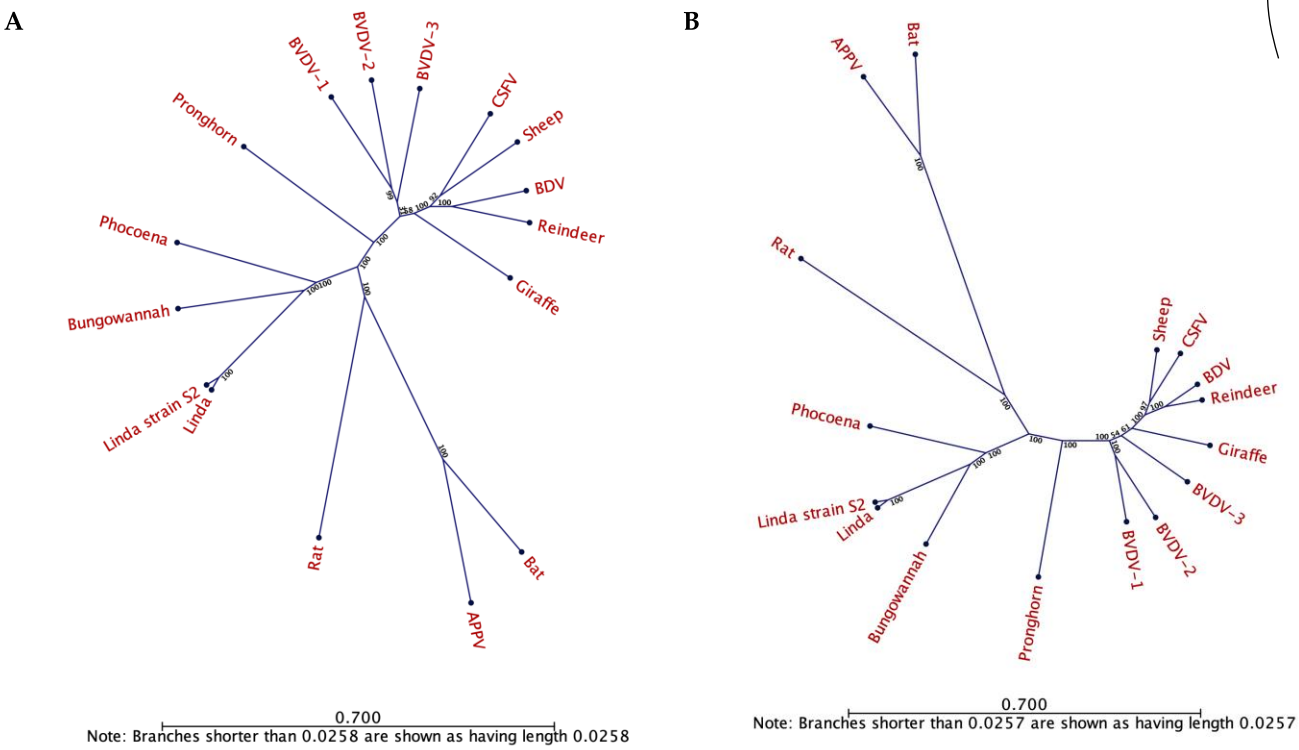

Figure 6. Phylogenetic analysis of the novel Linda virus strain S2 and approved and unclassified pestivirus species. Phylogenetic trees based on the full nucleotide sequence (**A**) and the polyprotein sequence (**B**) were constructed based on the neighbor-joining algorithm and bootstrap analysis with 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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Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the use of archived serum samples, that were sent in for diagnostic purposes or obtained within the frame of other research studies.

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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