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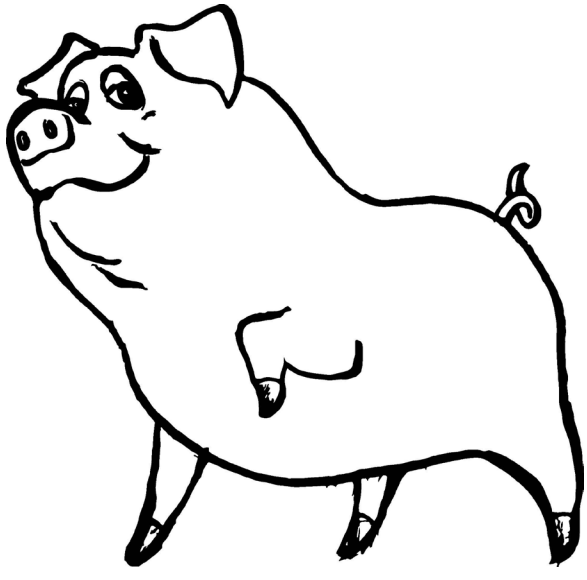
Congenital tremor and splay leg in Swedish piglets

– a study of the viral causes and routes of transmission

Hedvig Stenberg



Congenital tremor and splay leg in Swedish piglets – a study of the viral causes and routes of transmission

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Abstract

Congenital tremor (CT) and splay leg (SL) are diseases regularly seen in new-born piglets in Sweden. They are diagnosed based on the clinical picture, which in CT are tremor and sometimes ataxia, and in SL, a lack of adduction of the hind legs. The cause of the clinical signs is unknown, but impaired nerve function due to myelin damage is a theory. However, the syndromes are transient, and piglets that can nurse often recover within some months. For decades, there has been speculation about the causes of these diseases. Infection in utero with certain viruses, hereditary factors, and drug treatment of pregnant sows may induce the syndromes in the offspring. In Sweden, one type of CT has been described: CT type A-II, a variant that for a long time was suspected to be caused by an unknown virus. Then, in 2015, a new virus named atypical porcine pestivirus (APPV) was discovered, and in 2016, APPV was associated with CT type A-II. There were also indications of an association between APPV and SL. This thesis aimed to investigate viral causes of CT type A-II and SL in Swedish piglets. APPV was detected in brain tissue from piglets with signs of CT type A-II, both in new and historical material. The virus could also be detected in commercial boar semen from a Swedish boar stud and in serum samples from Swedish wild boars. However, the virus was not found in piglets with splay leg or in healthy piglets, and high-throughput sequencing of the material did not reveal any other relevant viruses. Consequently, it seems unlikely that APPV, or any other virus, would be the primary cause of SL in Sweden.

Keywords: APPV, pigs, congenital tremor, splay leg, virome, wild boar, artificial insemination

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Sammanfattning

Skaksjuka och fläksjuka är neurologiska sjukdomar som ses regelbundet hos nyfödda griskultingar i Sverige. Sjukdomarna diagnostiseras baserat på den kliniska bilden. För skaksjuka innebär det skakningar och ibland ataxi och i fallet med fläksjuka, bristande adduktion av bakbenen, ibland även av frambenen. Det är okänt vad som orsakar de kliniska symptomen men nedsatt nervfunktion på grund av myelinskador är en vanlig teori. Tillstånden är reversibla och kultingar som kan dia, blir normalt återställda inom ett par månader. Virusinfektioner, ärftliga faktorer och medicinbehandling av dräktiga djur har associerats till fläksjuka och till olika typer av skaksjuka. I Sverige förekommer skaksjuka av typ A-II, en variant som misstänktes vara virusorsakad men där viruset länge var okänt. År 2015 hittades ett virus som fick namnet Atypisk porcint pestivirus (APPV). APPV kunde associerades till skaksjuka typ A-II och det fanns indikationer om att det även orsakade fläksjuka. Målet med avhandlingen var att undersöka eventuella virala orsaker till skaksjuka typ A-II och fläksjuka hos griskultingar i Sverige. APPV påvisades i hjärnvävnad från griskultingar med skaksjuka, både i nytt och historiskt material. Viruset hittades inte hos griskultingar med fläksjuka eller hos friska griskultingar. Storskalig sekvensering av materialet visade inga relevanta virus förutom APPV hos grisar med skaksjuka. Att APPV eller något annat virus skulle vara den primära orsaken till fläksjuka i Sverige verkar därför inte troligt.

Nyckelord: APPV, Sverige, grisar, skaksjuka, fläksjuka, virom, vildsvin, artificiell insemination

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Dedication

To the piglets of the world, you deserve better.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. STENBERG, H.*, JACOBSON, M. & MALMBERG, M. 2020. Detection of atypical porcine pestivirus in Swedish piglets with congenital tremor type A-II. *BMC Vet Res,* 16**,** 260.

II. STENBERG, H.*, HELLMAN, S., LINDSTRÖM, L., JACOBSON, M., FOSSUM, C., HAYER, J. & MALMBERG, M. 2022. Congenital tremor and splay leg in piglets - insights into the virome, local cytokine response, and histology. *BMC Vet Res,* 18**,** 348.

III. STENBERG, H.*, LEVERINGHAUS, E., MALMSTEN, A., DALIN, A. M., POSTEL, A. & MALMBERG, M. 2021. Atypical porcine pestivirus-A widespread virus in the Swedish wild boar population. *Transbound Emerg Dis*, 4, 69.

IV. STENBERG, H.*, MALMBERG, M., HAYER, J. Screening for atypical porcine pestivirus in Swedish boar semen used for artificial insemination and a characterisation of the seminal RNA microbiome including the virome (Submitted manuscript)

*Corresponding author

Papers I-III are reproduced with the permission of the publishers.

The contribution of Hedvig Stenberg to the papers included in this thesis was as follows:

I. Planned the study together with the co-authors. Coordinated and performed the sampling, laboratory experiments, and data analysis. Wrote the manuscript with support from the co-authors.

II. Planned the study together with the co-authors. Coordinated and performed the sampling, laboratory experiments, and data analysis. Wrote the manuscript with support from the co-authors.

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Related publication

STENBERG, H., JACOBSON, M. & MALMBERG, M. 2020. A review of congenital tremor type A-II in piglets. Anim Health Res Rev, 1-5.

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1. Introduction

1.1 General background

Congenital tremor type A-II and splay leg are considered as neurological disorders affecting new-born piglets. The syndromes present with clear clinical signs, tremor and ataxia, or abduction of the limbs, respectively (Kinsley 1922; Thurley *et al.* 1967). Congenital tremor was described in 1922 by Kinsley, and splay leg in the year 1967 by Thurley et al. Although globally known for decades, the causative agents have been elusive, and several different causes and agents have been suggested and investigated (Miller *et al.* 1973; Jirmanova 1983; Ohnishi *et al.* 1989; Bölcskei *et al.* 1996; Sellier *et al.* 1999; Blomstrom *et al.* 2014; Jeong *et al.* 2017; Lamp *et al.* 2017; Wen *et al.* 2018). The clinical signs of congenital tremor type A-II and splay leg are often transient, however, case fatality rates up to 50% have been described.

In the year 2015, a novel virus was identified; Atypical porcine pestivirus (Hause *et al.* 2015) and the virus was quickly associated with congenital tremor type A-II (Arruda *et al.* 2016; de Groof *et al.* 2016; Postel *et al.* 2016). When atypical porcine pestivirus (APPV) was detected, congenital tremor in piglets had for many years generated a low interest. Following the detection of a presumptive causative agent, the interest in congenital tremor increased substantially.

When inoculation studies with APPV were performed, not only piglets with signs of congenital tremor were born but also some piglets with a co-morbidity of congenital tremor type A-II and splay leg (Arruda *et al.* 2016;

de Groof *et al.* 2016). Thus, it was speculated that APPV was the causative agent of both splay leg and congenital tremor type A-II (Arruda *et al.* 2016; de Groof *et al.* 2016), but as APPV was recently discovered, knowledge about the virus and its traits was lacking. Further, nothing was known about APPV in Sweden prior to the studies included in this thesis, except from a report of an incidental finding of a Porcine pestivirus with 90% similarity to APPV in a piglet with Post weaning Multisystemic Wasting Syndrome, PMWS (Blomstrom *et al.* 2016).

2. Background

2.1 Congenital tremor type A-II

Congenital tremor (CT) is a neurological syndrome in neonatal piglets induced during the embryonic/ foetal stage. The syndrome is characterised by tremor of the head and body, in severe cases complicated by ataxia (Kinsley 1922; Larsson 1955; Done 1968; Done 1976; Bolske *et al.* 1978; de Groof *et al.* 2016). Congenital tremor is divided into six subtypes; type A I – V and type B (Table 1). All six types of CT may initially present similarly, but the progression and lethality differ between the types. Today, Sweden holds a disease-free status for classical swine fever virus (WOAH 2022), and the hereditary and medical-induced types of CT are well-known and accordingly controlled (Done 1976; Bolske *et al.* 1978). Thus, as far as known, only congenital tremor type A-II and congenital tremor type B are present in Sweden.

Table 1. An overview of the types of congenital tremor

Type	**A-I**	**A-II**	**A-III**	**A-IV**	**A-V**	**B**
Cause	Classical swine fever virus	Atypical porcine pestivirus	Hereditary	Hereditary	Metrifonate-treatment of pregnant sow	Unknown, idiopathic? Probably not of infectious origin
Lethality	High	Low – medium	High	High	High	Low – medium
Sex	⚥	⚥	♂	⚥	⚥	⚥
Breed	All	All	Landrace	Saddleback	All	All
Transient	No	Yes	No	No	No	Yes
References	(Bradley et al., 1983)	(Patterson et al., 1976, Arruda et al., 2016, Postel et al., 2016)	(Done, 1976)	(Done, 1976)	(Bolske et al., 1978)	(Done, 1968, Done, 1976)

CT type A-II type has been known for a long time, and an unidentified virus was suggested as the causative agent in 1978 (Bolske *et al.* 1978). However, it was only recently a virus, atypical porcine pestivirus (APPV), was identified and associated with the disease (Arruda *et al.* 2016; de Groof *et al.* 2016; Postel *et al.* 2016). Congenital tremor type A-II occurs in domestic pigs globally, but the prevalence is not yet established (Done 1976). In China, it has been estimated that 1-2 % of all live-born piglets are affected by CT (Yuan *et al.* 2017). A Swedish study from 1986 that included 1716 individuals found an incidence of non-specific "trembling symptoms" of 11% among Land-race pigs and an incidence <1% among Yorkshire, Hampshire and Duroc pigs (Lindström & Lundeheim 1986). There are, however, no recent publications of estimates or studies on the prevalence of CT type A-II in Sweden or Europe.

Since horizontal infection with APPV does not induce clinical signs, transmission of CT type A-II among the piglets appears without prior notice (Done 1976; Postel *et al.* 2016; Stenberg *et al.* 2020a). Similar to the other types of congenital tremor, CT type A-II is characterised by tremor and sometimes ataxia (Arruda *et al.* 2016; de Groof *et al.* 2016; Stenberg *et al.* 2020a). The tremor is an action tremor, thus, evident when the piglets are standing or moving and absent when piglets are resting or sleeping. Thus far, there are no reports of fever or impaired general health associated with CT type A-II. However, the tremor and ataxia may affect the piglets' ability to nurse and move, which may increase the pre-weaning mortality due to malnutrition or injuries done by the sow.

Commonly, an outbreak of CT type A-II in a herd continues for 2 – 3 months, with a within-litter prevalence of < 10- 100 % (de Groof *et al.* 2016). Following an outbreak, the sows seem to develop immunity, and there may be several years until a new outbreak occurs, if ever. According to the literature, piglets born to primiparous sows are at risk of being affected by CT type A-II, as compared with piglets born to multiparous sows (Stenberg *et al.* 2020a). The case-fatality rate during an outbreak is generally low, but there are reports of losses up to 15-20% due to starvation or crushing by the sow (Bolske *et al.* 1978; Done *et al.* 1986; Stenberg *et al.* 2020a). Still, the syndrome is transient, and most piglets will have clinically recovered at

around three months of age (Arruda *et al.* 2016; de Groof *et al.* 2016; Schwarz *et al.* 2017; Cagatay *et al.* 2019).

No gross lesions have been described for CT type A-II, but the syndrome is associated with histological lesions such as mild hypomyelination and mild vacuolisation of the white matter of the central nervous system (Done 1968; Done 1976; Done *et al.* 1986; Duncan 1987). More recent reports of microscopical lesions associated with CT type A-II include everything from no lesions, mild and moderate vacuolisation, hypomyelination of the white matter of the brain, to severe lesions of the brain such as demyelination, oedema, neuronal necrosis, and Purkinje cell necrosis (Postel *et al.* 2016; Schwarz *et al.* 2017; Dessureault *et al.* 2018; Mósena *et al.* 2018; Possatti *et al.* 2018a; Possatti *et al.* 2018b; Sutton *et al.* 2019; Ren *et al.* 2022; Stenberg *et al.* 2022).

2.2 Splay leg

Splay leg, spraddle leg syndrome, or porcine splay leg syndrome (PCS) is characterised by muscle weakness and impaired function of the hind limbs that causes a varying degree of uncontrolled lateral abduction (Thurley *et al.* 1967). Sometimes, the front and hind limbs are splayed. In that severe case, the piglet will be completely immobilised. The impaired mobility associated with splay leg affects the piglets' ability to nurse and leads to hypoglycaemia and malnutrition, as well as an increased risk of maternal crushing (Schumacher *et al.* 2021). Further, splay leg may predispose to arthritis and pododermatitis due to abrasions associated with reduced mobility.

Splay leg is transient, and most piglets improve within a week. Still, there are reports of lethality up to 50% (Zelená & Jirmanová 1979; Ooi *et al.* 2006; Schumacher *et al.* 2021). However, if given appropriate care, such as proper nutrition and supportive bands around the affected hind legs to enable mobility, the piglet survival rate will increase significantly (Ooi *et al.* 2006; Schumacher *et al.* 2021).

Approximately 0.4 % of the live-born piglets display signs of splay leg, and the within-farm prevalence ranges from 1–8%, commonly with 1-3 affected piglets per litter(Ward & Bradley 1980; Papatsiros 2012). Thus, splay leg is considered one of the most critical congenital diseases in modern pig production (Partlow *et al.* 1993).

Early studies revealed that splay leg was more prevalent in heavily muscled breeds compared with other more gracile breeds of pigs (Dobson 1968; Vogt *et al.* 1984; Tomko 1993), indicating a hereditary predisposition of the disease (Lax 1971). Since then, several studies have tried to disclose the genetic background of splay leg, and some candidate genes have been suggested (Maak *et al.* 2003; Boettcher *et al.* 2007; Hao *et al.* 2017; Wu *et al.* 2018). In addition to the genetic predisposition, there are several risk factors associated with the syndrome, *e.g.,* insufficient intrauterine nutrition (Cunha 1968; Kanora & Maes 2009), exposure of pregnant sows to mycotoxins and medicines *e.g*., Zearalenone (Miller *et al.* 1973), glucocorticoids (Jirmanova 1983), or pyrimethamine (Ohnishi *et al.* 1989; Jeong *et al.* 2017), induction of partus with prostaglandins (Bölcskei *et al.* 1996; Sellier *et al.* 1999), and viral infections such as PRRSV or APPV

during pregnancy (Arruda *et al.* 2016; de Groof *et al.* 2016; Jeong *et al.* 2017).

The full pathogenesis of splay leg is not yet understood, and the aetiology is suggested to be multifactorial (Ooi *et al.* 2006). Although readily diagnosed in the clinic, histopathological diagnosis is not straightforward, and a range of non-pathognomonic lesions are associated with splay leg. The most commonly described lesion is myofibrillar hypoplasia, which is an immaturity of the skeletal muscle fibres(Thurley *et al.* 1967; Dobson 1968; Zelená & Jirmanová 1979; Ward & Bradley 1980; Jirmanova 1983) also seen in healthy neonatal piglets (Hanzlikova 1980; Ducatelle *et al.* 1986; Curvers *et al.* 1989; Schumacher *et al.* 2021). Still, there are piglets with clinical signs of splay leg without detectable myofibrillar hypoplasia. Interestingly, there seem to be many mechanisms behind myofibrillar hypoplasia, e.g., in natural cases of splay leg the extra myofibrillar space is glycogen-filled, whereas piglets born with medically induced myofibrillar hypoplasia display only a small amount of glycogen in the extra myofibrillar space (Ducatelle *et al.* 1986).

Some authors have associated splay leg with hypomyelination of the nerves innervating the hind limbs and lumbar spinal cord (Szalay *et al.* 2001; Papatsiros 2012), and degenerative changes in the muscle fibres of the hind legs (Zelená & Jirmanová 1979). Yet, there are also piglets with signs of splay leg without detectable hypomyelination or degenerative changes (Bradley *et al.* 1980). As for the clinical signs, the lesions are reversible. Piglets with signs of splay leg that can nurse will show clinical and histological recovery from the myofibrillar hypoplasia, hypomyelination, and degeneration of muscle fibres around one week of age (Zelená & Jirmanová 1979; Szalay *et al.* 2001).

2.3 Pig production in Sweden

For decades, there have been a trend towards larger herds and fewer farms in Sweden (Official Statistics of Sweden 2023). Many farms have adopted an integrated farrow-to-finish regime. Thus, sow pools and the trade with live animals have become less common. The number of pigs has also decreased. During the 1980ies, there were almost two times as many pigs in Sweden compared to the current number of ~140 0000 pigs. During the same period, the number of Swedish pig farms decreased from 21 100 to 1070 farms (Official Statistics of Sweden 2023). See Figure 1 for an overview of the development of the number of pigs and farms over the last 13 years.

Figure 1. The number of Swedish pigs and pig farms from 2010 to 2022

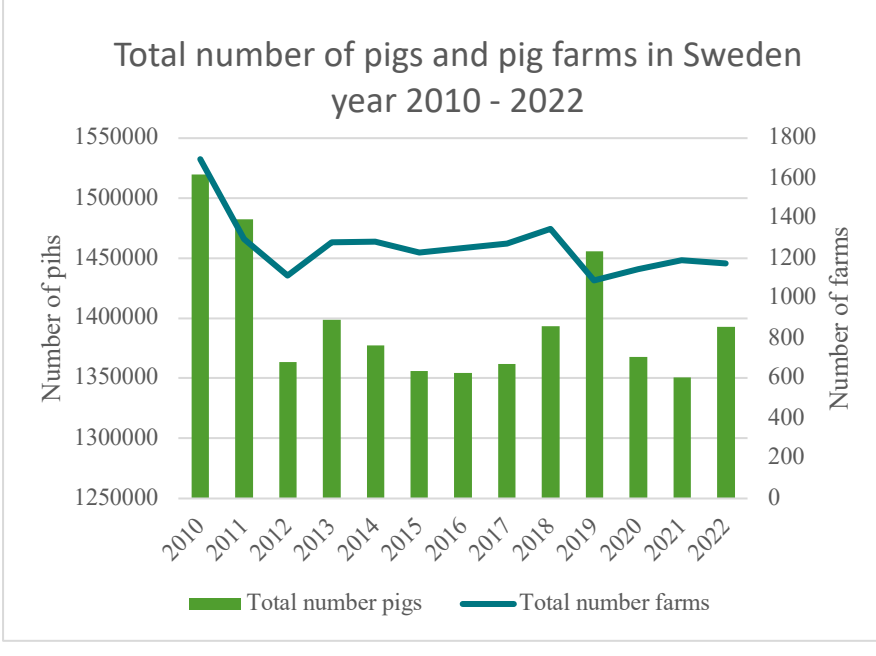


Figure 1. The figure displays the number of pig and pig farms in Sweden from the year 2010 to 2022. The numbers are recorded yearly on the first Thursday in June (Official Statistics of Sweden 2023)

The market share for Swedish pork, which dropped after Sweden joined the EU, has steadily increased over the few last years. In 2021, 82.7% of the pork consumed in Sweden was of Swedish origin (Gård&Djurhälsan 2022). In 2022, ~120,000 sows produced 2.67 million pigs for slaughter, a slight increase from 2021 (Official Statistics of Sweden 2023). Of the slaughtered pigs, 2.6% were bred in organic farms with outdoor access.

Most pig farms are located in the south or central parts of Sweden (Figure 2). A majority of the pigs are housed indoors, in pens with one-part slatted floor and one-part solid floor. According to Swedish legislation (SJVFS 2019:20), all pigs should be provided with straw or equivalent bedding material (Jordbruksverket, 2019). Farrowing crates are prohibited. The average weaning age in 2021 was 33 days (Gård&Djurhälsan 2022). Male pigs are castrated either by surgery within their first week of life or by medical "immuno-castration". Tail docking and teeth cutting are prohibited, however, teeth grinding is allowed under specific circumstances (Jordbruksverket 2019). Following weaning, the pigs are moved to growing units, holding 10 -12 pigs per pen. Some litters are kept intact although, regrouping and cross-fostering are common. After weaning, sows are commonly kept in groups on deep-litter straw beds in un-insulated stables during breeding and gestation.

The health status of the Swedish pig population is considered good (Statens jordbruksverk 2021; National veterinary institute 2022). The most common diagnoses for the treatment of piglets pre-weaning are arthritis, neonatal diarrhoea, and "unspecified disease of the cloves" (Gård&Djurhälsan 2021). There is no national vaccination programme for pigs in Sweden, but vaccination against porcine parvovirus, erysipelas (*Erysipelothrix rhusiopathiae),* Porcine circovirus type 2 (PCV2), and neonatal diarrhoea *(E. coli)* is recommended. Then, dependent on the health status of the specific farm, recruitment strategy, and demands from buyers of grower pigs, additional vaccinations may occur against *e.g.,* Porcine influensa virus, *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae*, Lawsonia *intracellularis*, and *Clostridium perfringens* type A and C (National veterinary institute 2022).

Figure 2. The distributions of wild boars and pigs in Sweden in the year 2022

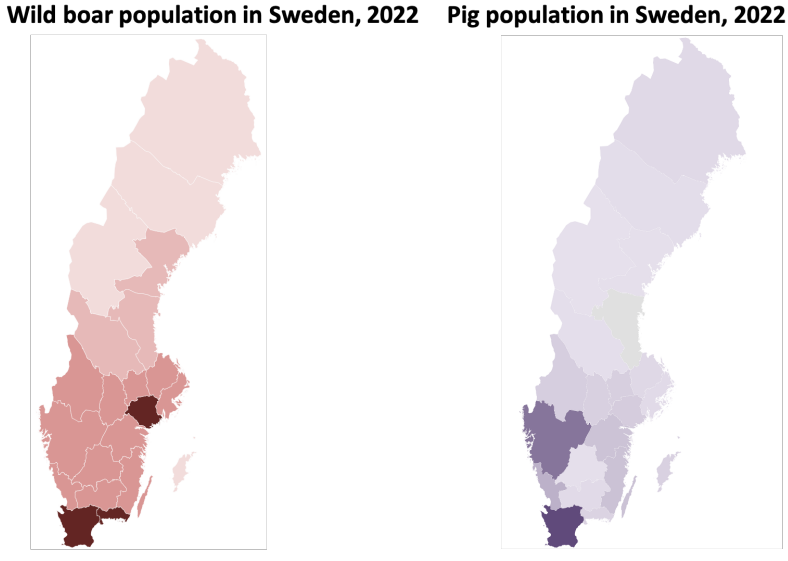


Figure 2. The maps show the distributions of wild boars and pigs in Sweden in 2022 based on reported shootings (Viltövervakning 2023) and the reported number of pigs in Sweden in 2022 (Official Statistics of Sweden 2023). The dark colouring of the map represents the densest population of wild boars and domestic pigs, respectively. The densest wild boar populations are found in the counties where the wild boars first were established. In the south of Sweden, these wild boar-dense counties overlap with areas of intensive pig farming (Official Statistics of Sweden 2023). These are also the counties where *S.* cholerasuis has been detected (Ernholm *et al.* 2022) and some of the high-risk areas for African swine fever virus introduction into the Swedish wild boar population (Chenais 2021).

In Sweden, most producers (~70 %) record their production figures in the PigWin software (Gård&Djurhälsan 2022). These figures are then used for individualised farm advisory services as well as an annual report comparing the results across several countries in the EU within the interPIG collaboration project (Gård&Djurhälsan 2022). Taken together, the results of the Swedish pig farms are placed in the middle compared with other EU interPIG countries. Across the EU countries sampled, Sweden stands out with the highest pre-weaning mortality (16.9%) but with mortality among rearing and finishing pigs clearly below average (Gård&Djurhälsan 2022).

The average number of pigs weaned per sow and year in the EU interPIG countries was 29.7 in 2021 compared to 27.9 pigs weaned per sow and year in Sweden (Gård&Djurhälsan, 2022). However, the piglets in Sweden are weaned a few days later compared with the other InterPig countries.

Artificial insemination with fresh, chilled, and extended semen is the dominant breeding technique, practised in almost 100% of Swedish pig farms. The commercial trade of semen includes four breeds; Hampshire, Yorkshire, Landrace, and Duroc (Norsvin & DanAvl). Generally, the fattening pigs are crosses of two or three breeds, sows are either pure-breed or a two-breed crossing, and breeding boars are pure-breed (Gård&Djurhälsan 2022).

One company, Svenska Köttföretagen AB, is responsible for coordinating the trade and production of semen for artificial insemination, as well as the trade of live animals for breeding. At any given time, there are 400 approved boars for semen production and a turnover of approximately 50 new boars from quarantine every sixth week. The breeding boars are kept under a specific pathogen control program. The control program included treatment with anthelmintics, blood sampling for monitoring, and vaccination against some listed pathogens (see paper IV for details). The breeding boars are bred in Sweden or Norway.

New genetics are primarily acquired through semen, however, there is also a limited import of pigs to Sweden. In 2020, a total of 215 pigs were imported: one from Finland and 90 from Norway for breeding purposes, in addition to one zoo-river pig from Germany and 123 pigs from Denmark for production (Official Statistics of Sweden 2023).

2.4 Wild boars in Sweden

2.4.1 Wild boar occurrence in Sweden and the Nordic Countries

During the last decades, the European wild boar (*Sus scrofa*) population has steadily been increasing, and the population in Scandinavia has expanded towards the north (Melis *et al.* 2006; Hedmark *et al.* 2021; Maaroufi *et al.* 2022). Historical documents and archaeological excavations have shown that wild boar used to be part of the fauna in Sweden and Norway before eradication due to hunting and domestication (Lemel & Truvé 2008; Rosvold *et al.* 2010). In Sweden, free-roaming wild boar was absent from the 1700s century until the 1970'ies when a few individuals escaped enclosures. In 1981, the Swedish government decided that wild boar were invasive and should be eradicated from Sweden (1980/81:JoU 16) but, in 1988, a policy decision (1986/87:JoU 15) was made to accept wild boar as part of the Swedish fauna (Lemel & Truvé 2008). The wild boars were first established in the county of Skåne and Södermanland (Figure 2 and Figure 3), and have spread from there. Today, the most dense populations are still found in these counties (Lemel & Truvé 2008; Naturvårdsverket 2020). Wild boars have a low niche conservatism and a high invasive potential (Sales *et al.* 2017). Thus, it is speculated that with climate change and unregulated supplementary feeding, the wild boar will keep expanding north along the East Coast and the hinterland until it reaches the mountains (Hedmark *et al.* 2021).

Figure 3. Map of Sweden

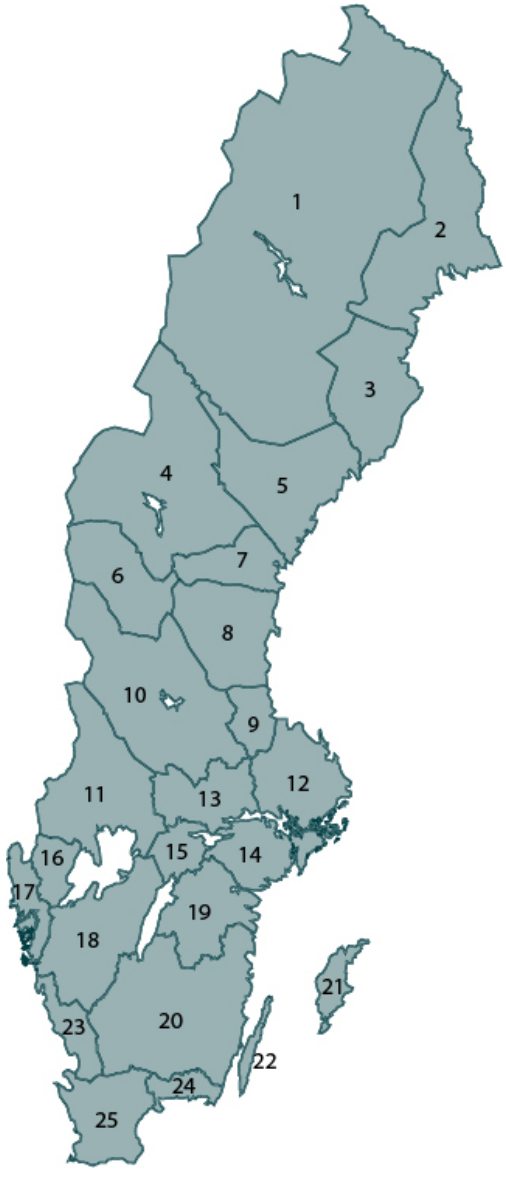


Figure 3. The counties of Sweden from the north to the south: 1. Lappland, 2. Norrbotten, 3. Västerbotten, 4. Jämtland, 5. Ångermanland, 6. Härjedalen, 7. Medelpad, 8. Hälsingland, 9. Gästrikland, 10. Dalarna, 11. Värmland, 12. Uppland, 13. Västmanland, 14. Södermanland, 15. Närke, 16. Dalsland, 17. Bohuslän, 18. Västergötland, 19. Östergötland, 20. Småland, 21. Gotland, 22. Öland, 23. Halland, 24. Blekinge, and 25. Skåne.

There is no organized counting of wild boars and the population size-approximation is based on the hunters' voluntary reporting of shootings. In 2020, the population size was estimated to be at least 300, 000 individuals (Naturvårdsverket 2020). This is in contrast to the wild boar populations in the other Nordic countries; in Norway, there are ~ 400 -1 200 wild boars, in Denmark < 5, and in Finland ~ 1 500 - 3 400 (VKM 2018; Hedmark *et al.* 2021). There are reported contacts between wild boars at the border of Norway and Sweden but not between wild boars in Sweden and Denmark, or Finland.

A free-roaming, unregulated wild boar population is controversial as it is associated with negative impacts such as the spread of diseases, destruction of habitats and crops via rooting, traffic accidents, competition and predation on wildlife (Lemel & Truvé 2008; VKM 2018; Hedmark *et al.* 2021; Kilgo *et al.* 2021). There are also reports on off-springs from mating between domestic pigs and wild boars (Jori *et al.* 2017) and studies show that pig farms in areas where wild boars are present may have regular wild boar visits (Wyckoff *et al.* 2009; Wu *et al.* 2012; Bacigalupo *et al.* 2022). In Sweden, there are recent reports of pathogen transmission between wild boars and domestic pigs (Stenberg *et al.* 2021; Ernholm *et al.* 2022), in addition to anecdotal reports of mating between domestic pigs and wild boars.

In 2020, a decision was made to intensify hunting pressure and make easements in the hunting regulation of wild boars to decrease their impact on agriculture and traffic by half by 2025 (Naturvårdsverket 2020; Hedmark *et al.* 2021). Thus far, this has resulted in fewer traffic incidents involving wild boars, and the hunting bag of 2021 and 2022 has decreased due to the assumed decrease of the wild boar population, Figure 4 (Viltövervakning 2023).

Figure 4. Yearly shootings of wild boars from the year 1990 to 2021

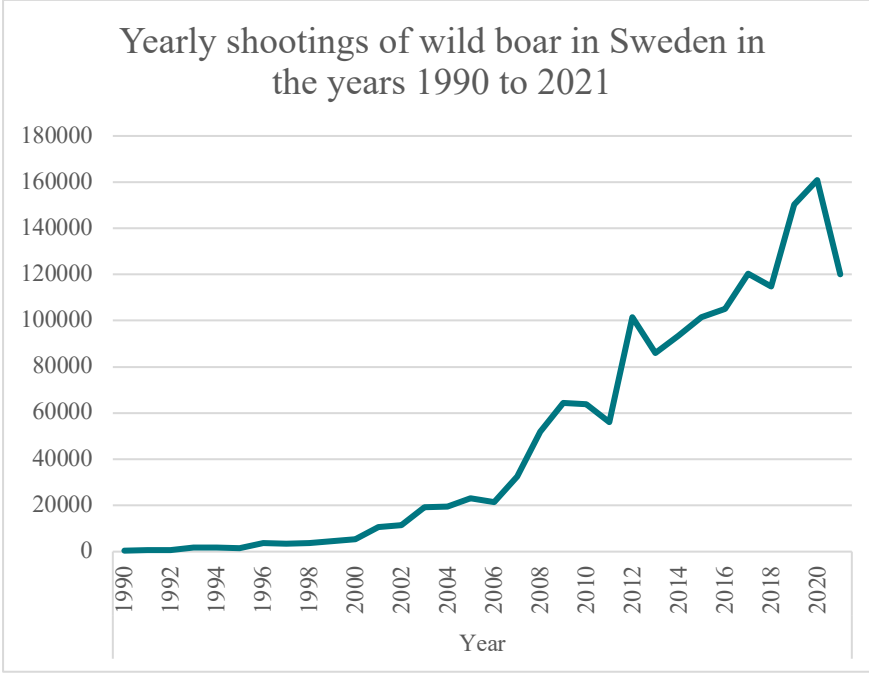


Figure 4. The reported yearly shootings of wild boars are used to estimate the number of wild boars in Sweden (Viltövervakning 2023). The decreased number of wild boars shot in the year 2021 indicates a reduction in the population.

Wild boars are nocturnal and live in sounders with females and piglets. The male wild boars live in solitary but generally interact with a higher number of other wild boars, both male and female, compared with wild boars that live in a sounder (Jordbruksverket 2010; Kilgo *et al.* 2021; Schlichting *et al.* 2022). However, young wild boars, < 2 years old, have the most between-group contacts (Podgórski *et al.* 2018). The home range varies from 1 - 10 km^2 depending on the available food resources and the population density within the area (Jordbruksverket 2010; Kilgo *et al.* 2021; Schlichting *et al.* 2022). These factors also affect how much sounders interact with each other (Jordbruksverket 2010; Kilgo *et al.* 2021; Schlichting *et al.* 2022). Thus, during the winter season, when the feed is limited, the sounders' home ranges overlap more than they do during the rest of the year (Schlichting *et al.* 2022).

2.4.2 Wild boar and pathogens

Domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa*) have high genetic similarity and may crossbreed (Groves 1981; Jonsson 1986; Mona *et al.* 2007; Iacolina *et al.* 2019). They are also susceptible to the same pathogens. This makes wild boar an important reservoir of pathogens, *e.g.*, African and classical swine fever virus (Artois *et al.* 2002; Meng *et al.* 2009; Sauter-Louis *et al.* 2021), hepatitis E virus (Schlosser *et al.* 2014; Schlosser *et al.* 2015), porcine circovirus (Segalés *et al.* 2005; Patterson & Opriessnig 2010; Rose *et al.* 2012), Suid herpesvirus 1 (SuHV1) (Müller *et al.* 2011), *Sarcoptes scabiei* (Sannö *et al.* 2021), *Toxoplasma gondii* (Olsen *et al.* 2019; Dubey *et al.* 2020), *Campylobacter* spp. (Wahlström *et al.* 2003; Castillo-Contreras *et al.* 2022; Kerkhof *et al.* 2022), *Salmonella* spp. (Fredriksson-Ahomaa *et al.* 2020; M *et al.* 2020; Papić *et al.* 2021; Ernholm *et al.* 2022). Further, wild boar may also carry pathogens of zoonotic potential, such as hepatitis E virus, *Mycobacterium tuberculosis, Brucella suis, Yersinia* spp*., Salmonella* spp*., Toxoplasma,* and *Trichinella* spp. (Meng *et al.* 2009; Fredriksson-Ahomaa 2019). They may also transfer bacteria carrying antimicrobial resistance genes between domestic pigs as well as to the environment (Marotta *et al.* 2020; Plaza-Rodríguez *et al.* 2020; Torres *et al.* 2020; Darwich *et al.* 2021; Formenti *et al.* 2021; Ramos *et al.* 2022; Rega *et al.* 2022; Torres *et al.* 2022), *Eimeria* spp. (Petersen *et al.* 2020).

Sweden currently has a status of the domestic pigs as being free from several epizootic pathogens, *e.g.*, classical and African swine fever virus, porcine reproductive and respiratory syndrome virus (PRRSV), and pseudorabies virus (National Veterinary Institute, 2020). However, given the situation in Europe, a recent commission on wild boar in the Nordic countries, ordered by The Nordic Council of Ministers, ranked African swine fever as the most critical disease in wild boar for the porcine industry (Hedmark *et al.* 2021).

2.5 Pestivirus

The genus pestivirus belongs to the family *Flaviviridae*. It comprises well-known viral species of high global economic and clinical importance in addition to newly discovered pestivirus such as the atypical porcine pestivirus (Simmonds *et al.* 2017). Traditionally, pestiviruses have been associated with severe disease in ruminants or pigs, *i.e.*, bovine viral diarrhea virus, classical swine fever virus, and border disease virus but through metagenomic analysis, new species of pestivirus have been detected in a variety of clinically healthy hosts such as bats, rodents, whales, and pangolin (Blome *et al.* 2017a; Postel *et al.* 2021). Currently, there is no known invertebrate host.

A revised pestivirus taxonomy based on letters rather than full names was proposed in the year 2017; Pestivirus A (Bovine viral diarrhea virus 1), Pestivirus B (Bovine viral diarrhea virus 2), Pestivirus C (Classical swine fever virus) and Pestivirus D (Border disease virus), in addition to seven new species; Pestivirus E (pronghorn pestivirus), Pestivirus F (Bungowannah virus), Pestivirus G (giraffe pestivirus), Pestivirus H (Hobi-like pestivirus), Pestivirus I (Aydin-like pestivirus), Pestivirus J (rat pestivirus), and Pestivirus K (Atypical porcine pestivirus) (Simmonds *et al.* 2017; Smith *et al.* 2017). In 2021, eight additional pestivirus species were proposed within the genus (Postel *et al.* 2021).

Pestiviruses are enveloped, spherical viruses with a diameter of about 40-60 nm (Laude 1979). Being enveloped, the virions have low general and thermal stability and are quickly inactivated by organic detergents and solvents (Depner *et al.* 1992). In contrast to other flaviviruses, pestiviruses are very resistant to low pH (Laude & Gelfi 1979; Maurer *et al.* 2004).

The pestivirus genome is a single-stranded, positive-sense RNA of ~11.3–13 (-16.5) kb length, encoding one single open reading frame (ORF) accompanied by the 5′- and 3′- untranslated regions (Collett *et al.* 1988; Meyers *et al.* 1989; Becher *et al.* 1998; Becher *et al.* 2014; Saltik *et al.* 2022). The polyprotein is processed into capsid protein (C), envelope proteins (E^{rns}, E1, and E2), and non-structural proteins (p7, N^{pro}, NS2, NS3 (NS2-3), NS4A, NS4B, and NS5B) (Rice 1990; Simmonds *et al.* 2017; Smith *et al.* 2017). The envelope protein E^{rns} and the non-structural auto protease N^{pro} are

immunoregulatory and unique to the pestivirus genus (Lindenbach & Rice 2003).

Pestiviruses gain entry into cells by receptor-mediated endocytosis by attaching to cell surface molecules (Wang *et al.* 2004). The protein E^{rns} and the E2 -protein specifically bind to receptors on the cell surface to enable entry (Li *et al.* 2017; Goraya *et al.* 2018). Cellular receptors such as complement regulatory protein 46 (CD46), heparan sulphate (HS), low-density lipoprotein (LDL) receptor, and disintegrin and metalloproteinase 17 (ADAM17) are important for pestivirus infection (Hulst *et al.* 2001; Qi *et al.* 2022). Utilizing these ubiquitous cell surface proteins/receptors, pestiviruses generally exhibit multicell- and tissue-tropic behaviour. Pestivirus may infect lymphocytes, macrophages, epithelial cells, neurons, oocytes in the reproductive tract, mucosal epithelia, the central nervous system, the bone marrow, the peripheral blood, and the lymphoid tissue (Hewicker-Trautwein *et al.* 1992; Marshall *et al.* 1996; Liebler-Tenorio *et al.* 1997; Wang *et al.* 2004; García-Pérez *et al.* 2009; Qi *et al.* 2022).

2.5.1 Transmission of pestiviruses

Pestiviruses are transmitted horizontally, directly or indirectly through infected body fluids e.g., saliva, faeces, urine, semen, milk, and respiratory droplets (Meyling et al. 1990; Choi & Chae 2002; Tautz et al. 2015) as well as vertically (Plateau et al. 1980; Stewart et al. 1980; Gardiner & Barlow 1981; de Groof et al. 2016). The transmission of Classical swine fever virus (CSFV), Bovine Viral Diarrhea Virus (BVDV), and Border Disease virus (BDV) usually occurs via the oronasal route through direct contact with infected animals (Nettleton et al. 1998; Lanyon et al. 2014; Schweizer & Peterhans 2014; Blome et al. 2017b). Although viruses are shed in abundance in faeces, the orofaecal route is deemed to be of secondary importance for the transmission (Brownlie et al. 1987).

2.5.2 Clinical signs associated with pestiviruses

The clinical outcome of the infection is dependent on the age and immune status of the animal and may result in subclinical disease, persistent infection, severe illness, or death (Baker 1995; Tautz *et al.* 2015). Infection

in utero may cause embryonic death and abortion, persistent infection in the offspring, teratogenic effects resulting in congenital abnormalities as well as a healthy, non-infected foetus, dependent on the time-point and gestation length (Grahn *et al.* 1984; Trautwein *et al.* 1986; Carlsson *et al.* 1989; Brownlie *et al.* 1998; Nettleton *et al.* 1998; Braun *et al.* 2002; García-Pérez *et al.* 2009; Otter *et al.* 2009; Porter *et al.* 2010).

Persistently infected animals are of high clinical relevance because they may be clinically undetectable while serving as a major viral reservoir, shedding virus in all body secretions (Brock 2003; Schweizer & Peterhans 2014; Tautz *et al.* 2015; Khodakaram-Tafti & Farjanikish 2017). Thus, direct contact between naïve and persistently infected animals is the most common route of infection (Lanyon *et al.* 2014; Blome *et al.* 2017b). In a bovine population lacking a BVDV control program, the estimated prevalence of persistently infected animals was ~1% (Houe 1995).

2.5.3 Persistent infections

In the case of pestivirus, persistent infections are the consequence of transplacental, foetal infections during the first trimester (Carbrey *et al.* 1980; Brownlie *et al.* 1998; Moennig *et al.* 2003; Lanyon *et al.* 2014; Blome *et al.* 2017b). Persistent infections are the result of the modulation of the host immune response and the inhibition of the induction of type I-interferons (Charleston *et al.* 2001; Peterhans & Schweizer 2013). The suppressed production of type I-interferons enables viral survival in the host by inhibiting the stimulation of important downstream antiviral actions (Baron & Dianzani 1994). This induces a peripheral tolerance towards the virus that alters the immune system (Schweizer *et al.* 2006; Hansen *et al.* 2010; Peterhans & Schweizer 2013). Persistently infected animals are viraemic, antibody-negative, and shed a high amount of virus (Brock 2003; Schweizer & Peterhans 2014; Tautz *et al.* 2015; Khodakaram-Tafti & Farjanikish 2017).

In addition to the persistent/prenatal form of a pestivirus infection, there is also a chronic and fatal form of classical swine fever following a horizontal infection with CSFV in juvenile animals (Blome *et al.* 2017b). The chronic form of CSF is attributed to an inadequate immune response in the adult

individual (Jenckel *et al.* 2017) and is associated with non-specific clinical signs such as intermittent fever and wasting (Moennig *et al.* 2003).

Immunocompetent and healthy individuals who manage to mount an appropriate immune response may suffer from a transient viremia that causes a subclinical or clinical disease followed by immunity to the infection (Nettleton *et al.* 1998; Moennig *et al.* 2003; Lanyon *et al.* 2014). The infected animals mainly produce antibodies against the envelope proteins E^{rns}, E2, and the NS2-3/NS3 protein, however, antibodies towards E^{rns} and E2 proteins are the primary neutralizing antibodies (Weiland *et al.* 1990; van Zijl *et al.* 1991; Weiland *et al.* 1992; König *et al.* 1995; Reimann *et al.* 2004).

Due to the antigenic relatedness of the "classical" pestiviruses, cross-reactivity between different species may occur, inducing protective immunity and interference with laboratory diagnostics (Carbrey *et al.* 1976; Deregt *et al.* 1994; Tautz *et al.* 2015; Huang *et al.* 2021; Bohórquez *et al.* 2022; Muasya *et al.* 2022). The newly discovered pestiviruses are more genetically distinct from the classical pestiviruses and also from each other *(Hause et al. 2015; Simmonds et al. 2017; Smith et al. 2017; Meyer et al. 2021; Postel et al. 2021)*. Thus far, no false positive results have been obtained when CSFV or BVDV RNA have been tested in an APPV-specific PCR (Postel *et al.* 2017b; Kaufmann *et al.* 2019). Further, the APPV RNA did not induce cross-reaction in serological antigenic assays for the foot-and-mouth disease virus, swine vesicular disease virus, porcine respiratory and reproductive syndrome virus, or African swine fever virus (Kaufmann *et al.* 2019). APPV-specific antibodies do not cross-react with routinely applied serological tests for diagnosis of pestiviruses such as border disease virus (BDV), classical swine fever virus (CSFV), Aydin-like pestivirus, or Bungowannah pestivirus (BuPV) (Postel *et al.* 2017b; Meyer *et al.* 2021).

2.5.4 Pestivirus control

Among the pestiviruses, classical swine fever and bovine virus diarrhoea are endemic in large parts of the world and pose threats to the livestock and pig industry globally (Richter *et al.* 2019; Nielsen *et al.* 2021). Effective control measures differ between the two diseases, mainly due to the ability of BVDV

to cause persistently infected (PI) calves that may be clinically difficult to detect (Ridpath *et al.* 1994; Ståhl & Alenius 2012; Moennig & Becher 2015).

Vaccination as a control measure for BVDV is discussed and has been regarded as an important practical measure against disease associated with the virus (Sandvik 2004). Thus far, vaccination has not been able to change the epidemiological situation of BVDV, mainly due to the insufficient induction of foetal protection (O'Rourke 2002). Fortunately, control and eradication of BVDV have proven efficient using a non-vaccination approach (Lindberg & Alenius 1999). When targeting persistently infected individuals and removing the source of foetal infection, in combination with implementation of high biosecurity and continuous monitoring of free herds, within 10 years, the Scandinavian countries were free or almost free from BVDV (Sandvik 2004; Hult & Lindberg 2005; Rikula *et al.* 2005; Ståhl & Alenius 2012; Løken & Nyberg 2013).

In the case of CSF, stamping out and vaccination have proven efficient in controlling and eradicating the disease (Kaden *et al.* 2005; Moennig 2015; Moennig & Becher 2015; Rossi *et al.* 2015). Oral immunisation with modified live vaccines is an efficient strategy for CSF control in free-ranging wild boars compared to alternative control strategies such as intensified hunting or fences (Kaden *et al.* 2005; Moennig 2015; Moennig & Becher 2015; Rossi *et al.* 2015). However, since the 1990ies, there has been a general EU ban on CSF vaccines and emergency vaccination must be approved by the commission. Since a majority of the EU countries currently hold a CSF-free status (WOAH 2022), the primary focus for controlling CSF within the EU is the implementation of strategies from early detection, in addition to high biosecurity inducing movement and trading control of pigs.

2.6 Atypical porcine pestivirus

Atypical porcine pestivirus was first detected by high-throughput sequencing during a Porcine Reproductive and Respiratory Syndrome (PRRS) characterisation project on clinically healthy pigs in the United States of America (Hause *et al.* 2015). Through experimental and natural infections, the virus was associated with congenital tremor type A-II in piglets (Arruda *et al.* 2016; de Groof *et al.* 2016; Postel *et al.* 2016). Like the other members of the genus Pestivirus, APPV is an enveloped, single-stranded, positive sense RNA virus and part of the *Flaviviridae* family. Today, APPV is the only member of the species Pestivirus K and the name Pestivirus K has been suggested instead of Atypical porcine pestivirus (Simmonds *et al.* 2017; Smith *et al.* 2017; Postel *et al.* 2021).

The APPV virion is spherical with a diameter of approximately 60 nm (Liu *et al.* 2020). The genome size is approximately 11-11.6 kb and has a 5′-noncoding region (NCR), one open reading frame (ORF), and a 3′-NCR region, Figure 5 (Hause *et al.* 2015). The polyprotein consists of 12 proteins: capsid protein C, envelope proteins E^{rns}, E1, E2, and nonstructural proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Hause *et al.* 2015; Blome *et al.* 2017a; Simmonds *et al.* 2017). The level of amino acid identity compared to other pestiviruses varies between the proteins; E^{rns}, NS3, NS4B, and NS5B have the highest nucleotide conservation (20 – 29 %), whereas N^{pro}, p7, NS2, and NS5A have a nucleotide identity < 5% (Riedel *et al.* 2021).

The highest genome similarity is seen between APPV and a pestivirus isolated from Chinese bats (*Rhinolophus affinis*) with 68% genome identity (Hause *et al.* 2015). However, a low genome similarity (25 – 28%) is seen compared to other, well-known, pestiviruses such as bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus (Hause *et al.* 2015). Despite the distinctions, APPV uses the same conserved host cell receptor, CD46, as BVDV for the initial entry into macrophages and lymphocytes of the tonsils prior to being systemically distributed (Liebler-Tenorio *et al.* 1997; Maurer *et al.* 2004; Cagatay *et al.* 2021; Qi *et al.* 2022). CD46 is a transmembrane protein expressed on many cell types (Liszewski & Atkinson 1992; Ni Choileain & Astier 2012) and used for cell entry by several viruses and bacteria (Cattaneo 2004).

Figure 5. Schematic images of the APPV virion and genome

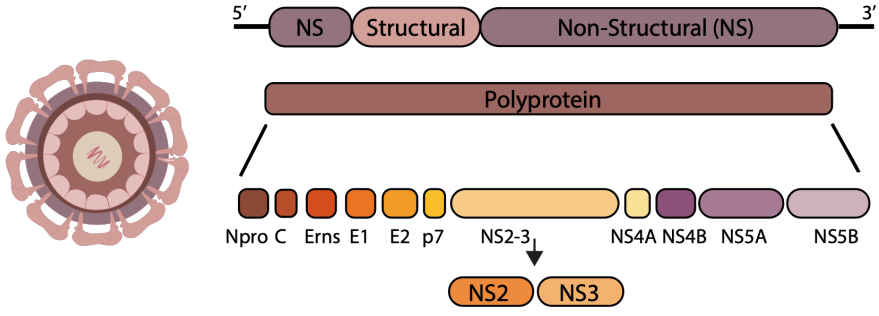


Figure 5. The APPV virion is enveloped and spherical. The genome has a 5′-noncoding region, one open reading frame, and a 3′-noncoding region. It encodes for a polyprotein consisting of 12 proteins. The picture of the genome is modified from Tautz et al. (2015) and Hause et al. (2015).

Today, there is no consensus on what part of the APPV genome is most suited for PCR detection or phylogenetic analysis but the NS3 and NS5B coding regions, as well as the 5'-untranslated region (5' UTR), have been suggested and used for this purpose (de Groof *et al.* 2016; Beer *et al.* 2017; Schwarz *et al.* 2017; Yuan *et al.* 2017; Cagatay *et al.* 2018; Mósena *et al.* 2018; Kaufmann *et al.* 2019; Sozzi *et al.* 2019; Folgueiras-González *et al.* 2020b; Grahofer *et al.* 2020; Stenberg *et al.* 2020b; Riedel *et al.* 2021; Zhang *et al.* 2021; Arruda *et al.* 2022; Sutton *et al.* 2022; Yuan *et al.* 2022). However, the APPV-genome is highly variable, also within a small geographical area, and the NS3 and NS5B coding regions, regarded as relatively conserved, may remain undetected when not using genome sequencing (Sutton *et al.* 2022).

Three genotypes of APPV have been proposed (Beer *et al.* 2017; Pan *et al.* 2018; Xie *et al.* 2019; Yan *et al.* 2019; Yuan & Wang 2021; Zhang *et al.* 2021; Kasahara-Kamiie *et al.* 2022; Ren *et al.* 2022; Yuan *et al.* 2022) and there is a suggestion of a fourth genotype or sub-group (Choe *et al.* 2020). The highest genome variability is reported in China, and two of the proposed genotypes (I and II) have only been detected there (Pan *et al.* 2020; Zhang *et al.* 2021; Ren *et al.* 2022; Mou *et al.* 2023). Further, there are reports of interclade recombination events in China (Guo *et al.* 2020).

2.6.1 Clinical signs, viremia, shedding, and viral persistence

Intrauterine infection with APPV is associated with congenital tremor type A-II, and an association between APPV and splay leg combined with congenital tremor type A-II has been suggested (Arruda *et al.* 2016; de Groof *et al.* 2016; Postel *et al.* 2016; Stenberg *et al.* 2020a).

No clinical signs of disease have been described following horizontal infection or postnatal inoculation with APPV, only a transient viremia has been recorded (Arruda *et al.* 2016; de Groof *et al.* 2016; Cagatay *et al.* 2019; Sutton *et al.* 2019; Folgueiras-González *et al.* 2020b; Grahofer *et al.* 2020). However, following transplacental infections, prolonged viremia has been recorded in piglets born with congenital tremor and in their healthy littermates (de Groof *et al.* 2016; Cagatay *et al.* 2019). In addition, these studies also reported viremia that persisted until the time of slaughter, despite clinical recovery.

Persistent infections with APPV have been suggested following the detection of viraemic but clinically healthy pigs displaying high antibody levels (Postel *et al.* 2017a) and pigs shedding the virus in body fluids such as faeces, saliva, and semen several months after the clinical recovery (de Groof *et al.* 2016; Postel *et al.* 2016; Schwarz *et al.* 2017; Yuan *et al.* 2017; Gatto *et al.* 2018a; Buckley *et al.* 2021). Furthermore, intermittent shedding of APPV in faeces after viral clearance in blood has also been reported (de Groof *et al.* 2016; Folgueiras-González *et al.* 2020b). Persistently APPV-infected animals and prolonged viral excretion would agree with the infectious pattern recorded for other pestiviruses (Randall & Goodbourn 2008; Tautz *et al.* 2015).

2.6.2 Pathology

Gross examination of piglets infected with APPV and with signs of CT type A-II are generally without significant findings (Done *et al.* 1986; Schwarz *et al.* 2017; Liu *et al.* 2019; Stenberg *et al.* 2020b). Histological examinations, however, have described a varying degree of hypomyelination and vacuolisation (very mild to severe) of the white matter of the cerebrum, cerebellum, and spinal cord (Done *et al.* 1986; Postel *et al.* 2016; Lamp *et al.* 2017; Schwarz *et al.* 2017; Possatti *et al.* 2018b; Stenberg *et al.* 2020b). There are also descriptions of APPV-positive piglets exhibiting clear signs

of CT type A-II but without histopathological alterations of the brain, such as vacuolisation of the white matter, or hypomyelination (Postel *et al.* 2016; Dessureault *et al.* 2018; Stenberg *et al.* 2022). A compilation of the reported histopathological lesions seen in piglets with signs of congenital tremor type A-II is provided in Table 2.

One transmission electron microscopy (TEM) study on the cerebellar white matter, cerebellar peduncles, and medulla oblongata from two CT-affected new-born piglets is available (Schwarz *et al.* 2017). TEM examination revealed ultrastructural defects such as mild hypomyelination and disruption and breakdown of the myelin and intramyelinic vacuoles containing membranous debris. Other ultrastructural defects were observed, such as membrane-bordered vacuoles within axons and the cytoplasm of glial cells (Schwarz *et al.* 2017).

The cerebellum

The cerebellum is crucial for regulating motor functions such as balance, coordination, and posture (Manto *et al.* 2012; Perciavalle *et al.* 2013; Caligiore *et al.* 2017; Lang *et al.* 2017). Thus, if the nerve function of the cerebellum is impaired, *e.g.*, as a result of infection or degradation, severe movement disorders may occur. Injuries to the cerebellum play a central role in several movement disorders, especially ones that include signs of tremor, ataxia, or dystonia (Kumar *et al.* 2022).

As the cerebrum, the cerebellum starts to develop in the first trimester (Amore *et al.* 2021) and has a massive postnatal development (Knickmeyer *et al.* 2008). Postnatal brain development is characterised by synaptogenesis, gliosis, and myelination (Rice & Barone 2000). In pigs, the cerebellum and cerebrum have reached ~95% of their maximum volume 20-23 weeks after birth (Conrad *et al.* 2012).

The cerebellar cytoarchitecture is made up of an internal medulla containing nerve fibres, supporting neuroglial cells, small blood vessels, and an external cortex organised in three layers (Consalez *et al.* 2020). The three laminae of the cortex include a molecular layer, a Purkinje cell layer, and a granular layer. The following cell types are included in the cortex; Purkinje cells,

Golgi cells, stellate cells, and basket cells are inhibitory neurones; granule cells and unipolar brush cells are excitatory neurones (Geurts *et al.* 2003; Consalez *et al.* 2020; Amore *et al.* 2021). The outer molecular layer is made up of the dendritic trees of the Purkinje cell dendrites, basket cells, and stellate cells, making synapsis with the parallel fibres from the granule cells. In the middle layer or Purkinje cell layer, the cell body of the Purkinje cells is located, extracting their dendrites through the inner granular layer and to the outer molecular layer. These cells provide the output of the cerebellar cortex. The inner granular layer is the input layer of the cerebellum and includes the granule cells, the Golgi cells, and the synapsis in this layer include the descending dendrites of granule and Golgi cells, and the ascending mossy fibres from the brain stem and spinal cord (Geurts *et al.* 2003; Reeber *et al.* 2013; Consalez *et al.* 2020; Amore *et al.* 2021).

Classically, ataxia is associated with cerebellar degeneration. In post-mortem examination of the cerebellum from human patients with ataxia, loss of Purkinje cells is commonly seen (Louis *et al.* 2019). Essential tremor, a neurological disorder characterised by rhythmic shaking of the head, trunk, and extremities, is associated with abnormal synaptic connections of the climbing fibres and Purkinje cells of the cerebellum (Lin *et al.* 2014; Kuo *et al.* 2017). The climbing fibres originate from the inferior olive of the medulla oblongata. Each climbing fibre innervates one Purkinje cell, making this synaptic connection exact but also vulnerable (Barmack & Yakhnitsa 2011; Lin *et al.* 2014).

Table 2. A compilation of the reported histopathological lesions seen in piglets with signs of congenital tremor type A-II

Cerebellum	**Cerebrum incl. Truncus encephali**	**Spinal cord**	**Other organs**	**Staining**	**Reference**
A few vacuoles in the white matter of the cerebellum and moderate hypomyelination.	Mild, focal gliosis. Partially perivascular.	Moderate hypomyelination of the white matter. Mild, focal gliosis. Partially perivascular. Thickness reduction of the myelin sheaths. A few dilated myelin sheaths and vacuoles in the white matter of the medulla oblongata and spinal cord.		HE & LFB	(Schwarz *et al.* 2017).
A few vacuoles in the white matter.	Severe oedema of the brainstem.	Hypomyelination and vacuoles in the white matter.	Severe oedema of lymphocytes in the submaxillary lymph nodes. Inflammatory cell infiltration and	HE & LFB	(Ren *et al.* 2022)

Cerebellum	Cerebrum incl. Truncus encephali	Spinal cord	Other organs	Staining	Reference
			cellulose exudation in the alveolar capillaries or alveolar cavities of the lung.		
A few vacuoles in the white matter	A few vacuoles in the white matter of the brainstem			HE	**(Sutton *et al.* 2019)**
Myelin vacuolization with formation of digestion chambers in the white matter. Mild secondary demyelination.	Multifocal neuronal necrosis, neuronophagia and gliosis in the cerebral cortexes. Myelin vacuolization with formation of digestion chambers in the white matter. Reduction of the thickness of myelin sheaths, severe	A mild loss of myelin accentuated in the lateral white matter Multifocal neuronal necrosis, neuronophagia and gliosis. Myelin vacuolization with formation of digestion chambers in the white matter.		HE, LFB & CV	(Possatti *et al.* 2018b)

Cerebellum	Cerebrum incl. Truncus encephali	Spinal cord	Other organs	Staining	Reference
	secondary demyelination with complete absence or an inadequate amount of myelin in both the white and grey matter of the brainstem.	Reduction of the thickness of myelin sheaths, severe secondary demyelination with complete absence or an inadequate amount of myelin in both the white and grey matter.			
Vacuoles in equal numbers in the white matter of the brain and spinal cord in healthy piglets and in piglets with signs of congenital tremor or splay legs.	Vacuoles in equal numbers in the white matter of the brain and spinal cord in healthy piglets and in piglets with signs of congenital tremor or splay legs.	Vacuoles in equal numbers in the white matter of the brain and spinal cord in healthy piglets and in piglets with signs of congenital tremor or splay legs.		HE, LFB & CV	(Stenberg *et al.* 2022)
Moderate vacuolization of the white matter.				HE	(Mósena *et al.* 2018)
		Mild reduction of myelin in in the lateral	Mild suppurative omphalitis.	HE & LFB	(Postel *et al.* 2016)

Cerebellum	Cerebrum incl. Truncus encephali	Spinal cord	Other organs	Staining	Reference
		white matter of the spinal cord.			
Neuronal necrosis, gliosis, neuronophagia, satellitosis, demyelination, Wallerian degeneration, and Purkinje cell necrosis.	Neuronal necrosis, gliosis, neuronophagia, satellitosis, demyelination, Wallerian degeneration, and Purkinje cell necrosis.	Neuronal necrosis, gliosis, neuronophagia, satellitosis, demyelination, Wallerian degeneration, and Purkinje cell necrosis.		HE	(Possatti *et al.* 2018a)
No changes were noted in the cerebral cortex, cerebellum, and brainstem of affected piglets.	No changes were noted in the cerebral cortex, cerebellum, and brainstem of affected piglets.	A few random vacuoles in both lateral and ventral funiculi (white matter). Oligodendrocytes appeared more prominent compared with the control piglet. Hypomyelination in the white matter of the spinal cord.		HPS & LFB	(Dessureault *et al.* 2018)
The main gross at necropsy and	The main gross at necropsy and	The main gross at necropsy and		HE	(Zhang *et al.* 2017)

Cerebellum	Cerebrum incl. Truncus encephali	Spinal cord	Other organs	Staining	Reference
histological detection showed no significant findings.	histological detection showed no significant findings.	histological detection showed no significant findings.			
Moderate vacuolization of the white matter.				HE	(Mósena *et al.* 2018)
		Mild reduction of myelin in in the lateral white matter of the spinal cord.	Mild suppurative omphalitis.	HE & LFB	(Postel *et al.* 2016)

Table 2: In the table, abbreviations of the staining names are stated. Their full names are; Hematoxylin and Eosin stain (HE), Luxol Fast Blue stain (LFB), Cresyl Violet stain (CV), Hematoxylin Phloxine Saffron stain (HPS).

The HE is the standard staining when performing histopathologic evaluations. The LFB is used to observe myelin in the CNS. The CV is used to stain the Nissl substance in the neurons and the cell nuclei in the CNS tissue. The LBF and the CV are often used together when evaluating the CNS tissue. The HPS is similar to the HE, but the HPS stains collagen yellow instead of pink as the HE does.

2.6.3 APPV-related immunity

Since APPV has been detected relatively recently, details concerning the viral mechanisms and immunity toward the virus are unknown, and there are only a few studies available on the topic. While the details concerning APPV immunity are uncharacterised, there is a consensus that APPV may induce a protective immunity following infection that can prevent vertical transmission. Thus, congenital tremor is mainly seen in litters born to gilts, assumed to be naïve (Postel *et al.* 2016; Gatto *et al.* 2018b; Possatti *et al.* 2018a; Folgueiras-González *et al.* 2020b; Pedersen *et al.* 2021) and following an outbreak of CT at a farm, piglets with signs of CT may not be seen for several years (Grahofer *et al.* 2020).

The APPV genome encodes two immunoregulatory proteins; N^{pro} and E^{rns}, unique to the members of the *Flaviviridae* family (Tautz *et al.* 2015). As interferon antagonists, N^{pro} and E^{rns} partly inhibit the activation of the innate immune system, thus enabling infection. Although the N^{pro} region of APPV has a low amino acid identity compared to other pestiviruses and seems to lack a crucial zinc-binding ability (Szymanski *et al.* 2009), indications are that APPV N^{pro} still possesses specific IRF-3 antagonistic activity (Mou *et al.* 2021). The Interferon regulatory factor 3 (IRF-3) is a central transcription factor of the interferon system that, when being upregulated, activates the type-I IFN pathway (Randall & Goodbourn 2008; Liu *et al.* 2015). The type-I interferons (Isaacs & Lindenmann 1987) include a large group of molecules such as IFN-α, -β, -ω, -ϵ, -τ, -δ and -κ. The IFN-α and IFN-β are the proteins directly involved in the antiviral response, acting on receptors leading to the stimulation of cellular antiviral genes (Baron & Dianzani 1994; Randall & Goodbourn 2008).

Further, a recent study confirmed that the APPV E^{rns} protein, in parallel to other pestiviruses, may inhibit interferon expression (de Martin & Schweizer 2022). The E^{rns} protein prevents the production of immunostimulatory pathogen-associated molecular patterns, PAMPs (Iqbal *et al.* 2004; Magkouras *et al.* 2008) by RNase cleavage of the viral ss- and dsRNA (Windisch *et al.* 1996; Iqbal *et al.* 2004; Mätzener *et al.* 2009). The absence of viral PAMPs (ss- and dsRNA) inhibits downstream signalling pathways that stimulate the innate immune response by producing inflammatory

cytokines, type I interferons (IFN), and other molecules (Kawasaki & Kawai 2014).

From other pestiviruses, it is known that the envelope glycoproteins E^{rns}, E2, and the non-structural protein 3 (NS3) induce a detectable humoral immune response following a natural infection (Bolin & Ridpath 1989; Chimeno Zoth & Taboga 2006; Wang *et al.* 2015; Cagatay *et al.* 2019). Similar antibody response is associated with APPV infections (Postel *et al.* 2017a; Postel *et al.* 2017b; Schwarz *et al.* 2017; Cagatay *et al.* 2018; Cagatay *et al.* 2019; Michelitsch *et al.* 2019; Yan *et al.* 2020; Arruda *et al.* 2022).

Similar to other pestiviruses (Weiland *et al.* 1990; Weiland *et al.* 1992; König *et al.* 1995), the neutralising capacity of serum seems to correlate with the presence of APPV E2-specific antibodies and not with the levels of E^{rns}-specific antibodies (Cagatay *et al.* 2019). However, following a horizontal infection, consistent levels of neutralising antibodies against both E2 and E^{rns} antigens have been observed (Cagatay *et al.* 2019). In the study, antibody titres remained high and neutralising after infection for >160 days in piglets and for about seven weeks in adult animals (Cagatay *et al.* 2019). Maternally derived antibodies (MDA) of a low to moderate neutralising capacity, were detected in most piglets with signs of CT and in their healthy littermates.

Transplacentally infected piglets with signs of CT type A-II generally display more individual and variable antibody kinetics compared with horizontally infected animals. The antibody titres of piglets with signs of CT are overall lower, faster declining, and reactive towards either E2 or E^{rns} antigen (Cagatay *et al.* 2019). However, this seemingly inadequate immune response may still clear an APPV infection (Folgueiras-González *et al.* 2020b). Folgueiras-González et al. (2020) described how piglets with signs of CT type A-II and with low levels of APPV-specific antibodies had cleared the virus from serum once they reached adulthood.

2.6.4 Detection of APPV by serology

Currently, there is no commercial serology for APPV but there are several in-house ELISAs for the E^{rns}, E2, and NS3 antigens (Postel *et al.* 2017a; Postel *et al.* 2017b; Schwarz *et al.* 2017; Cagatay *et al.* 2018; Cagatay *et al.*

2019; Michelitsch *et al.* 2019; Yan *et al.* 2020; Arruda *et al.* 2022). In contrast to the "classical" pestiviruses, no cross-reactivity or interference with other pestiviruses has been seen (Postel *et al.* 2017b; Kaufmann *et al.* 2019; Meyer *et al.* 2021). The immune response to APPV- E^{rns}, E2, and NS3 antigens have been evaluated for diagnostic serology, displaying that the E^{rns} ELISA was the most suitable for diagnostic purposes. The APPV E^{rns} antibodies were detected earlier and for a longer time period compared to E2 and NS3 antibodies (Cagatay *et al.* 2019; Arruda *et al.* 2022). Compared with serum, saliva provided the most reliable results for antibody detection (Arruda *et al.* 2022).

2.6.5 Detection of APPV in tissue

APPV is distributed systemically in infected animals (Arruda *et al.* 2016; Postel *et al.* 2016; Schwarz *et al.* 2017; Yuan *et al.* 2017; Liu *et al.* 2019; Yan *et al.* 2019; Buckley *et al.* 2021; Ren *et al.* 2022). The virus has been detected in all tested organ systems, *e.g.*, the CNS, lymph system, gastrointestinal system, respiratory system, heart, liver, and urogenital system including testis tissue, and salivary glands. When comparing the viral loads in tissue, the cerebellum (Liu *et al.* 2019; Buckley *et al.* 2021) and serum (Pan *et al.* 2018; Ren *et al.* 2022) presented the highest viral loads. The high viral presence in the CNS tissue indicates that APPV readily passes through the blood-brain barrier. The viral genome is also abundantly present in saliva (Schwarz *et al.* 2017; Stenberg *et al.* 2020b; Arruda *et al.* 2022) and in lymphatic tissue such as lymph nodes (Pan *et al.* 2018; Liu *et al.* 2019; Buckley *et al.* 2021; Ren *et al.* 2022). Saliva also seems to have a higher sensitivity for APPV detection, both for genome and antibody detection, compared with serum (Arruda *et al.* 2022).

A comparison of the viral distribution in tissue obtained from piglets suffering from CT type A-II and in tissue obtained from adult boars recovered from CT displayed a similar distribution of APPV in the tissue (Buckley *et al.* 2021). Interestingly, the cerebrum and cerebellum contained the most viral RNA in clinically recovered adult pigs and in piglets with signs of CT type A-II (Buckley *et al.* 2021).

On a cellular level, transmission electron microscopy (TEM) and immunohistochemical detection of APPV have revealed that irrespective of the tissue, the virus was mainly found in the cytoplasm of cells (Postel *et al.* 2016; Liu *et al.* 2019; Buckley *et al.* 2021). Additionally, these studies demonstrated that the APPV primarily targeted nerve fibres in the CNS and reticuloendothelial cells in the lymphoid tissues (Liu *et al.* 2019; Buckley *et al.* 2021). The reticuloendothelial system, or the mononuclear phagocyte system (MPS), includes monocytes and macrophages of the lymph nodes, liver, spleen, lungs, bone marrow, and peritoneal cavity.

Using TEM, the APPV virions were evident within the inner granular cell layer of the cerebellum and in the cytoplasm of spinal ganglia neurons (Postel *et al.* 2016). This finding matches the results from immunohistochemical staining studies of the cerebellum where the nerve fibres/axons in the medulla/white matter, the granular, and molecular layers displayed intense positive staining (Liu *et al.* 2019; Buckley *et al.* 2021). The Purkinje cell layer and the molecular layer of the cerebellum showed no or less intense staining intensity (Liu *et al.* 2019; Buckley *et al.* 2021).

APPV cell tropism in the testes has also been researched. At present, two studies are available. One study discovered the most APPV-RNA in the tunica albuginea and a moderate viral presence in the lumen of seminiferous tubules. The viral distribution was similar in 2-day-old piglets with signs of CT type A-II and in clinically recovered boars (Buckley *et al.* 2021). The other study investigated the testes of new-born piglets with signs of CT type A-II (Dénes *et al.* 2021). This study detected APPV within the walls of medium-sized arteries, the testicular interstitium, and fusiform cells around the primitive, non-functional seminiferous tubules (Dénes *et al.* 2021). However, no viral RNA was found in the lumen of the seminiferous tubules. Thus, the results from these two studies are not congruent. In the study by Buckley et al. (2021), APPV was detected beyond the Sertoli cell barrier (blood-testis barrier), whereas Dénes *et al.* (2021) reported that no virus was detected in cells beyond the blood-testis barrier of the new-born piglets.

2.6.6 Routes of APPV transmission and pathogenesis

Studies have shown that APPV may be transmitted transplacentally as well as horizontally (Arruda *et al.* 2016; de Groof *et al.* 2016; Cagatay *et al.* 2019; Sutton *et al.* 2019; Folgueiras-González *et al.* 2020b; Houston *et al.* 2022). However, the exact routes of horizontal transmission are not yet fully understood. An orofecal transmission route has been suggested, given the prolonged detection and shedding of APPV in salivary glands, saliva, the gastrointestinal tract, and faeces (de Groof *et al.* 2016; Postel *et al.* 2016; Schwarz *et al.* 2017; Folgueiras-González *et al.* 2020b) as well as venereal transmission via semen, through both natural mating and artificial insemination (de Groof *et al.* 2016; Schwarz *et al.* 2017; Folgueiras-González *et al.* 2020b; Grahofer *et al.* 2020; Houston *et al.* 2022). The pathogenesis is currently not described, but lymphatic tissue, the cerebellum, and serum stand out with high viral loads following both vertical and horizontal transmission (Pan *et al.* 2018; Liu *et al.* 2019; Buckley *et al.* 2021; Ren *et al.* 2022)

Transplacental infection with APPV is associated with signs of congenital tremor in the off-spring through both experimental infections (Arruda *et al.* 2016; de Groof *et al.* 2016; Buckley *et al.* 2021) and natural infections (Postel *et al.* 2016; Gatto *et al.* 2018b; Pan *et al.* 2018; Cagatay *et al.* 2019; Gatto *et al.* 2019; Liu *et al.* 2019; Sutton *et al.* 2019; Folgueiras-González *et al.* 2020b; Guo *et al.* 2020; Stenberg *et al.* 2020b; Pedersen *et al.* 2021; Shi *et al.* 2021; Houston *et al.* 2022; Ren *et al.* 2022). A litter born to an APPV-infected dame may consist of a mix of uninfected, healthy piglets, infected and healthy piglets, and infected, diseased piglets. The concurrent occurrence of sick and healthy piglets in the same litter indicates a slowly progressing intrauterine infection (Cagatay *et al.* 2019).

There are also some reports of co-morbidity of congenital tremor and splay leg following infection with APPV (Arruda *et al.* 2016; de Groof *et al.* 2016; Schwarz *et al.* 2017; Gatto *et al.* 2018b; Houston *et al.* 2022) but the causality between APPV and splay leg are not established (Stenberg *et al.* 2020b; Stenberg *et al.* 2022). Further, there are no reports of piglets with APPV infection and solely splay leg as the clinical outcome.

2.6.7 Occurrence and prevalence of APPV

Although APPV recently was discovered, the virus is not new. Spatiotemporally evolutionary analyses place the most recent common ancestor (tMRCA) of the current APPV more than 100 years ago in Germany or the Netherlands (Yuan *et al.* 2017; Shi *et al.* 2021; Ma *et al.* 2022). The analyses further suggest a global viral migration through the transport of live swine (Yuan *et al.* 2017; Shi *et al.* 2021; Ma *et al.* 2022).

Thus far, APPV has been detected in sick and healthy animals in all continents where there are pigs, except Australia and Africa (Riedel *et al.* 2021). Comprehensive prevalence studies on APPV are lacking, and the reported detection rates of APPV vary from regions and countries and between studies. Currently, the earliest detection point is from archived porcine samples collected in 1986 (Kaufmann *et al.* 2019).

In clinically healthy pigs, the reported serum genome prevalence ranges from 2.4 – 22% in Germany (Postel *et al.* 2016; Beer *et al.* 2017; Michelitsch *et al.* 2019), 13 % in Switzerland, (Kaufmann *et al.* 2019), 13.9% in Spain (Munoz-Gonzalez *et al.* 2017), and 28.7% in the USA (Yuan *et al.* 2022). An overall on-farm genome prevalence of ~10% has been reported in Germany (Postel *et al.* 2016). Further, screenings of commercial boar semen in the USA showed that ~16% of the tested boars were shedding APPV (Gatto *et al.* 2018a; Chen *et al.* 2019).

Studies on the seroprevalence of APPV-specific antibodies displayed an individual seroprevalence of ~60% (1460 serum samples) in Europe and Asia (Postel *et al.* 2017a) and a within-farm seroprevalence of 37-46 % in Germany (1115 serum samples) (Michelitsch *et al.* 2019). Interestingly, the study from Germany by (Michelitsch *et al.* 2019) showed that the seroprevalence varied between farms; there were farms where all tested animals had APPV-specific antibodies and farms without a single seropositive pig.

2.6.8 APPV in wild boars

Atypical porcine pestivirus has not only been detected in domestic pigs, but also in wild boars in Spain (Colom-Cadena *et al.* 2018), Germany and Serbia

(Cagatay *et al.* 2018), South Korea (Choe *et al.* 2020), and Sweden (Stenberg *et al.* 2021). Studies on the occurrence of APPV in wild boars reveal a high seroprevalence of 52% in Germany (237/456), 67% in Serbia (10/15) (Cagatay *et al.* 2018), and 72% (433/595) in Sweden (Stenberg *et al.* 2021). The APPV-genome prevalence, as investigated in serum samples, varies more between countries, from quite high in Germany (19%, 87/465) (Cagatay *et al.* 2018) and Sweden (12%, 73/595) (Stenberg *et al.* 2021) to low in South Korea (0.008%, 18/2297), Spain (0.002%,1/437), and Italy (0,007%, 3/430) (Colom-Cadena *et al.* 2018; Sozzi *et al.* 2019; Choe *et al.* 2020).

Phylogenetic studies reveal a high genetic identity between APPV from wild boars and domestic pigs in Sweden and Italy (Sozzi *et al.* 2019; Stenberg *et al.* 2021), whereas a German study showed a genetic distinction between APPV from wild boars and domestic pigs (Cagatay *et al.* 2018). The South Korean study on wild boar detected both sequences with a high genetic identity between APPV from domestic pigs and wild boar, as well as sequences with a genetic distinction between wild boar and domestic pigs (Choe *et al.* 2020).

2.7 The virome

The virome represents the viral part of the microbiome in a given niche (Lecuit & Eloit 2013). The niche can be small or large, *e.g.,* defined as the faeces of one pig or a whole lake. At present, the human virome is the most researched and characterised. In comparison, knowledge of the porcine virome is scarce. Interestingly, a recent study of the porcine virome revealed that the assembly and in-body distribution of viruses are similar to the human virome (Shkoporov *et al.* 2022).

The virome is a relatively new concept (Anderson *et al.* 2003). Thus, it is less researched, and its role in maintaining homeostasis and health is probably underestimated compared to the bacterial part of the microbiome (Pyöriä *et al.* 2023). It has been shown that the destabilisation of an individual's virome is commonly associated with disease (Lim *et al.* 2016). Compared to the bacterial part of the microbiome, the virome is as diverse and stable over time but generally has a higher inter-individual variation (Reyes *et al.* 2012; Abeles *et al.* 2014; Virgin 2014; Abeles *et al.* 2015; Lim *et al.* 2016; Aggarwala *et al.* 2017; Mirzaei & Maurice 2017; Shkoporov *et al.* 2019; Shkoporov & Hill 2019; Liang & Bushman 2021). Furthermore, chronic carrier states or the incorporation of pathogenic viruses into the hosts' genome are frequent events that may benefit or harm the host (Norja *et al.* 2006; Barton *et al.* 2007; Furman *et al.* 2015; Bjornevik *et al.* 2022; Pyöriä *et al.* 2023).

Parallel to the last decades' development and use of high-throughput sequencing, knowledge of the virome has been expanding, and a high diversity of viruses has been found persisting within the human body (Turnbaugh *et al.* 2007; Virgin *et al.* 2009; Virgin 2014; Zou *et al.* 2016; Liang & Bushman 2021). While metagenomic sequencing has revealed that organs and tissues thought to be sterile contain a virome, characterising the complete microbiome is not straightforward. Experimental artefacts are always present in sequencing libraries, and caution must be taken when interpreting the results. Reagent contaminants, wrongly annotated genomes, and the impossibility of achieving a sterile sampling technique are just some examples that introduce false hits into the datasets (Salter *et al.* 2014b; Jervis-Bardy *et al.* 2015; Kim *et al.* 2017; de Goffau *et al.* 2018; Porter *et al.* 2021). In light of this, it is being discussed if low-biomass samples

originating from organs such as the CNS system (Kumata *et al.* 2020; Pyöriä *et al.* 2023), the amniotic fluid (Lim *et al.* 2018; Wang *et al.* 2022), or the placenta (Collado *et al.* 2016; de Goffau *et al.* 2019) harbours a microbiome including a virome, or if the detected virome really is part of the "kitome" or bioinformatical artefacts.

The mammalian virome can be subdivided into four; the eukaryotic virome, bacterial virome (bacteriophages), archaeal virome, and genetic elements of viral origin that can modify gene expression within host chromosomes *e.g.*, prophages, endogenous retroviruses, and endogenous viral elements (Virgin *et al.* 2009; Virgin 2014). Generally, bacteriophages dominate in the niches where bacteria are abundant (Wagner *et al.* 2013; Wylie *et al.* 2014; Rascovan *et al.* 2016; Shkoporov *et al.* 2019) whereas the eukaryotic virome is more commonly found within tissue (Chen & Hudnall 2006; Norja *et al.* 2006; Lautenschlager & Razonable 2012; Spandole *et al.* 2015; Imperiale & Jiang 2016; Berger *et al.* 2017; Pyöriä *et al.* 2017; Xu *et al.* 2021). Recent studies indicate that bacteriophages are the most abundant viruses in the mammalian virome. They are important for maintaining homeostasis by regulating the host bacterial communities in addition to acting as genomic reservoirs of bacteria as phages modulate the host's innate and humoral immune system in relation to bacteria, as well as directly protecting the host from bacteria by being present on the mucosa (Rascovan *et al.* 2016; Blanco-Picazo *et al.* 2020; Pyöriä *et al.* 2023; Wang *et al.* 2023).

Thus far, the tissue-resident virome of healthy individuals is not as well characterised compared to the virome of sick or dead individuals. Most data on the virome from healthy humans have originated from easily assessable body fluids or secretions and have focused on the DNA virome (Wylie *et al.* 2014; Adiliaghdam & Jeffrey 2020; Liang & Bushman 2021; Pyöriä *et al.* 2023). Viruses have been described in the cerebrospinal fluid from healthy humans (Pou *et al.* 2018; Edridge *et al.* 2019; Ghose *et al.* 2019; Blanco-Picazo *et al.* 2020; Neri *et al.* 2020; van Lieverloo *et al.* 2021) but, little is known about the tissue-resident virome of the brain and spinal cord (Kumata *et al.* 2020; Pyöriä *et al.* 2023).

When it comes to pigs, most studies on the porcine virome are restricted to investigations of the cause of disease, with a particular focus on the intestine

and faeces (Shan *et al.* 2011; Belák *et al.* 2013; Blomstrom *et al.* 2016; Blomström *et al.* 2018; Smoľak *et al.* 2022; Wu *et al.* 2022; Yang *et al.* 2022; Wang *et al.* 2023) in addition to a few studies on the porcine tissue virome in organs with a potential of xenotransplantation (Denner 2017; Denner 2022; Liu *et al.* 2023). In general, studies of the tissue-resident virome of healthy pigs are lacking. However, a recent study on the porcine virome of the gastrointestinal tract, liver, lung, and spleen suggests that the porcine virome is composed similarly to the human virome (Shkoporov *et al.* 2022). Thus, luminal samples from the intestine hold the highest diversity and loads of bacteriophages, mucosal samples had lower viral loads but a higher proportion of eukaryotic viruses, and the parenchymal organs contained some eukaryotic viruses and bacteriophages.

3. Aims

The work included in this thesis aimed to expand the knowledge on viral causes of congenital tremor type A-II and splay leg in Swedish piglets. The research questions that turned to specific aims arose while drafting a review of CT type A-II and following the detection of APPV in piglets with signs of CT type A-II.

The first aim was to establish if APPV was present in Swedish piglets with signs of congenital tremor or splay leg. Thus, piglets with signs of congenital tremor type A-II or splay leg were sampled and subjected to qPCR testing in addition to historical samples obtained from piglets with congenital tremor already available at the University (paper I).

The second aim was to study any naturally occurring viral co-infections with APPV in piglets with congenital tremor type A-II and any viral causes of splay leg, *i.e.*, the tissue-resident virome of the central nervous system (CNS) of sick and healthy piglets. RNA extracted from the brain and spinal cord tissue of congenital tremor type A-II, splay leg, and healthy piglets were subjected to high-throughput sequencing. The CNS tissue was also histopathologically evaluated, and the characterisation of a local cytokine response associated with APPV was initiated (paper II).

The third aim was to investigate the presence of APPV in the wild boar population and to start exploring wild boars as a potential reservoir for the virus. The study included serum samples archived in biobanks and collected between 2000 and 2018 for research and surveillance (paper III).

The final aim was to screen commercial boar semen intended for artificial insemination for the presence of APPV. By applying a high-throughput sequencing approach, simultaneous screening of the seminal virome and the microbiome could be performed (paper IV).

The APPV-specific aims were:

- To determine if APPV is present in Swedish piglets with signs of CT type A-II.
- To determine if APPV is present in Swedish piglets with signs of splay leg.
- To explore transmission routes for APPV by investigating the occurrence of APPV in commercial AI-semen from Swedish boars and the Swedish wild boar population.
- To characterise histopathological lesions in the brain associated with infection with APPV and signs of CT type A-II.
- To make a description of the local immune response of the brain in association with APPV infection.

Further, additional aims were to:

- To characterize the tissue-resident CNS-virome of piglets with signs of CT type A-II, splay leg, and healthy piglets.
- To characterize the boar semen microbiome, including the virome.

4. Comments on material and methods

The thesis comprises four papers describing studies performed on biological material from Swedish piglets, wild boars, and breeding boars (Figure 6). The following section contains a summary and comments on the material and methods used. The indicated manuscripts contain details of the used material and methods.

Figure 6. A flow chart of the samples analysed for this thesis

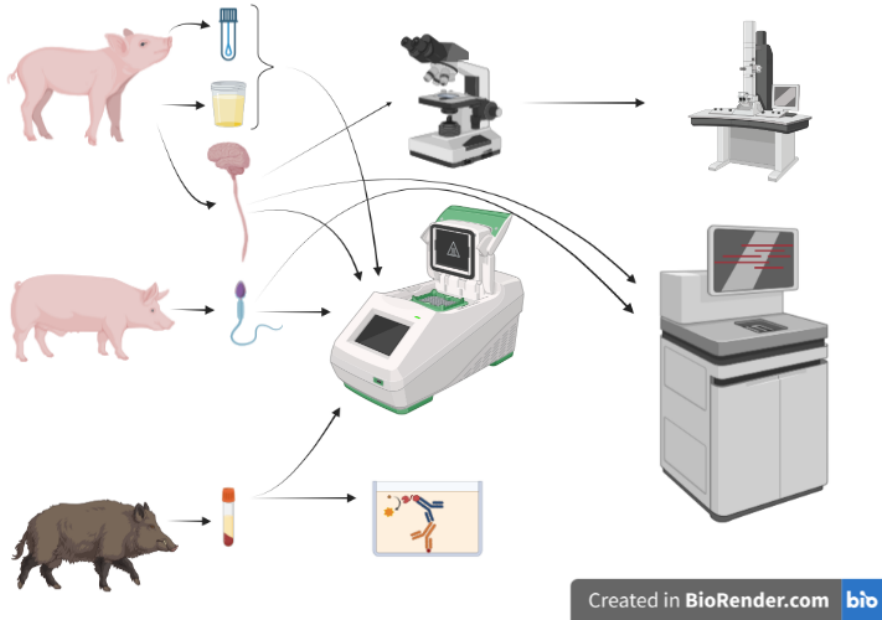


Figure 6. This figure presents a flow chart of the samples that were used in the work presented in this thesis.

4.1 Sampling of piglets

For the studies included in the thesis, piglets under five days old with clear signs of splay leg or congenital tremors were selected. The piglets originated from farms that provided healthy piglets for comparison. Attempts to recruit animals by advertisement in the Swedish porcine industry's monthly journal "Grisföretagaren" failed. Via veterinarians at the advisory company Gård och Djurhälsan (Farm and animal health service) and personnel at the Swedish University of Agricultural Sciences (SLU), contact was established with eight farms that either experienced an ongoing outbreak of congenital tremor or had at least one piglet with splay leg. The farms were located in the central and southern parts of Sweden (papers I and II) and the piglets were mainly sampled between June 2017 and 2018. In addition, archived material from piglets (n = 14) with signs of congenital tremor was included in the study.

All animal studies were performed following the current European (Directive 86/609/EEC) and the Swedish legislation on animal welfare (Djurskyddslag 2018:1192). The ethical committee of Uppsala 2017-02-10 (Dnr 5.8.10–00431/2017) approved the animal studies, and the farmers gave informed consent to the studies prior to selling the piglets. The ethical approval for this study sanctioned the use of a maximum of 50 piglets, but only 38 new-born piglets were euthanized and sampled within the present project. To maintain good animal welfare and to minimize transport stress for the animals, the piglets were co-transported only with littermates and piglets were mainly purchased from farms within three hours of travel time from Uppsala, Uppland. The five piglets from Blekinge were transported to the Section for Pathology at Gård & Djurhälsans facilities in Karlskrona, in the county of Blekinge, for a travel time of less than one hour. The option of on-farm euthanasia, sampling, and necropsy was dismissed to ensure accurate and quick handling of samples for viral metagenomics as well as pathology.

Piglets with signs of congenital tremor (n =15), piglets with signs of splay leg (n = 13), and healthy piglets (n = 8) were obtained. Two additional piglets with signs of congenital tremor were euthanized and sampled in January 2022 (paper II) for transmission electron microscopy. All piglets were

between 1 and 5 days of age. No more than five piglets with signs of disease were sampled at each farm.

The piglets with signs of congenital tremor were in good general condition. All were displaying signs equivalent to moderate or severe congenital tremor, with mild to moderate ataxia as defined by (de Groof *et al.* 2016). They were all born in litters where the piglets displayed a varying degree of tremor and ataxia. The piglets with signs of splay leg all had decreased general condition. The healthy piglets were in good health, showed no clinical signs of disease, and were chosen from litters where all piglets were born alive and healthy. None of the sampled farms had documented trading contact with each other or reported any simultaneous outbreaks or co-morbidity of congenital tremor and splay leg.

At the facilities, the piglets were anaesthetised with an intramuscular injection of tiletamine and zolazepam (Zoletil®, Virbac, Carros, France). Once sedated, blood samples were obtained from the jugular vein of the piglets. All piglets were euthanised by an intraperitoneal injection of pentobarbital (Allfatal vet. Apotek Produktion & Laboratorier AB, Malmö, Sweden). To ensure quick and correct handling of the piglets and to reduce the time from death to necropsy (one minute from confirmed cardiac arrest), the piglets were sedated and euthanised one at a time.

4.1.1 Sampling of tissue

For sampling, a protocol established to enable comparable and correct tissue-handling for the downstream analysis was used. Since the central nervous system is sensitive to post-mortem autolysis (Sheleg *et al.* 2008; Wohlsein *et al.* 2013; Tafrall 2019), the post-mortem examination began with the brain and spinal cord. The right brain hemispheres were frozen on dry ice and the left hemispheres were fixed in 10% formaldehyde. The spinal cords were subdivided into three; the cervical, thoracal and lumbar sections. Each section was split in two so that one part from the three sections was frozen and fixed in 10% formaldehyde. Once the CNS tissue was frozen and fixated, a complete post-mortem examination was performed. Samples from the brain, spinal cord, saliva, urine, heart, lung, quadriceps muscle, kidney, liver, spleen, ventricle, duodenum, jejunum, ileum, caecum, and colon were

sampled following a protocol developed to establish comparable and correct handling of tissue for the downstream analysis, put on dry ice, and fixed in 10% formaldehyde. After the initial freezing on dry ice, tissue samples were stored at − 80 °C and the corresponding tissue samples were fixed in 10% formaldehyde.

The instruments were changed between each organ system to minimize contamination. From five piglets originating from Södermanland, sampled in 2018, saliva and urine samples were also collected post-mortem using commercial E-Swabs (Copan Italia, Via Perotti, Italy).

For the histological evaluation, the brain was processed according to a standard excision schema used in the section for Pathology, SLU. Five sections of the brain were excised to cover the grey and white matter, the globus pallidus and putamen, parietal cortex, thalamus, hippocampus, mesencephalon, cerebellum, and obex.

4.1.2 Archived samples from piglets with signs of congenital tremor

Archived samples from 14 piglets with signs of congenital tremor and three healthy control piglets were also included in the qPCR screening for APPV (paper I). According to the records, the piglets displayed moderate to severe signs of congenital tremor. The archived samples included serum from eleven piglets with signs of congenital tremor sampled in 2004 and stored at − 80 °C, and RNA extracted from brain tissue originating from three piglets with signs of congenital tremor and three healthy control piglets sampled in late 2011 and early 2012, stored at − 80 °C (Blomstrom *et al.* 2014). All 14 piglets had been subjected to post-mortem examination but displayed no gross lesions.

4.2 Wild boar serum samples

A total of 595 serum samples from wild boars were included in the analysis. The serum samples were collected in 13 counties in the central and southern parts of Sweden (paper III) during hunting from 2000 to 2018, and stored in a biobank since. As the samples originated from wild boar from the ordinary

hunting bag, there is an element of convenience sampling to the collection. The number of sampled wild boars varied between year and county. However, since the serum sampling occurred during the hunt, counties with a dense wild boar population contributed with a higher number of sampled wild boars compared with the counties that harbour a sparser wild boar population.

Of the analysed samples, 464 were collected within the wild boar surveillance program run by the National Veterinary Institute. The blood was collected by hunters from the thoracic cavity of the wild boars using sterile blood sampling tubes and sent to the bio-bank at the National Veterinary Institute for serum separation and storage at −80 °C. The additional 131 serum samples originated from female wild boars shot in 2013 and 2014 and were collected within a PhD project. The blood was sampled from the thoracic cavity or the jugular vein using sterile blood sampling tubes, centrifugated, and the serum was stored in a −20 °C freezer as described in detail by Malmsten et al. (2017).

All samples included data on the sampling year, but some lacked further metadata. The serum samples from 2000 (n = 19) lacked information on the sampling location, and the samples from 2000 to 2001 (n = 13) lacked data on the date and location. Of the 595 serum samples, 89% (n = 531) had a recorded county of sampling, 68 % (n = 402) had a recorded sampling date, and 66% (n = 395) of the samples had records of the sampling year, county, and season. The missing metadata affected the statistical analysis. Still, trends of a varying proportion of APPV viraemic and seropositive wild boars over the years, seasons, and counties were seen.

4.3 Semen samples from breeding boars

In total, semen samples from 124 boars were analysed (paper IV). The samples consisted of extended, ready-to-use AI-doses in bags, each containing 80 mL, purchased from one of the Svenska köttföretagen AB's two boar studs in Sweden. At this boar stud, approximately 250 active breeding boars are kept at any given time, with a turnover of 25 new boars every sixth week.

The AI-doses were purchased in two batches. First, 24 AI-doses in January 2019 for metagenomic sequencing, followed by 100 AI-doses in October 2021 for a follow-up screening of APPV in semen. In all cases, one AI-dose represents one individual boar. All doses arrived in a climate box at SLU in Uppsala. The first 24 doses consisted of AI-doses from six Landrace boars, six Yorkshire boars, six Hampshire boars, and six Duroc boars. At the time of the follow-up screening for APPV in semen, only semen from Hampshire boars was available for purchase. Upon arrival at SLU, the semen was pipetted into tubes and stored at − 80 °C.

4.4 Pathology

All piglets included in this thesis were subjected to a post-mortem examination. Since the CNS was the main focus of the study, the brain and spinal cord were evaluated and sampled first to allow for a quick fixation and a reduced risk of post-mortem artefacts.

Five sections from the brain and three from the spinal cord were excised following a standardised protocol as described by Bolon et al. (2013) and used in the section of Pathology at SLU. The brain sections include the following anatomical structures: the caudate and putamen, cerebral cortex, corpus callosum, anterior commissure, septal nuclei, internal capsule, external capsule, hypothalamus, amygdala, thalamus, hippocampus, cerebral peduncles, optic tract, midbrain, pons, pyramids, cerebellum, reticular formation, trigeminal nuclei, medulla oblongata, and the choroid plexus. The three sections from the spinal cord included the cranial cervical segment, lumbar intumescence, and thoracic segment. The tissue samples were placed in a 10% neutral buffered formalin solution for fixation, paraffin-embedded, sectioned at 4 µm, mounted onto slides, and stained with haematoxylin and eosin.

Additional staining to evaluate the loss of myelin, neuronal chromatolysis of Nissl substance, and the potential presence of amyloid in the brain were performed. For this purpose, sections of the cerebellum and spinal cord from the three piglets with the highest viral load and one control piglet were stained using luxol fast blue and cresyl violet stain. Sections of the cerebrum

and cerebellum from the same piglets were stained with Congo red. The Congo red staining was done since recent research has suggested that viral infection of the brain associated with alterations of myelin, such as vacuolisation, may catalyse aggregation of the amyloid β-peptide in brain tissue (Ezzat *et al.* 2019; Malmberg *et al.* 2020).

4.5 Transmission electron microscopy (TEM)

To study ultrastructural lesions in the brain associated with APPV and congenital tremor type A-II, tissue from the cerebellum of two piglets, one with clinical signs of congenital tremor and one healthy control piglet, was subjected to TEM (paper II). The cerebellum was chosen for evaluation since previous reports described the most severe findings there (Schwarz *et al.* 2017; Mósena *et al.* 2018; Possatti *et al.* 2018b), as well as higher viral loads of the cerebellum compared to the cerebrum (Liu *et al.* 2019; Buckley *et al.* 2021).

Tissue from the cerebellum was cut into cubes of 1 mm^3 before fixation in 2.5% glutaraldehyde and 1% paraformaldehyde in PIPES. The embedding, preparation, sectioning, and visualization of the sections were done at the BioVisPlatform EM of Uppsala University (described in detail in paper II).

4.6 RNA extraction

RNA was extracted from a variety of tissues; the grey matter of the brain (frontal lobe), spinal cord, serum, saliva, urine, and semen using the same pre-preparation, extraction kit and protocol. The RNA extraction protocol we used for this project is commonly used in our lab and has previously been shown to produce RNA suitable for RT-qPCR and high-throughput sequencing.

During extraction, precautions were taken to avoid potential cross-contamination between samples and groups (healthy, congenital tremor, and splay leg) and to ensure an intact cold chain. Samples from the different groups (sick/healthy) were prepared separately, and no more than eight

samples were processed at the time in the lab. When the RNA was intended for high throughput sequencing, a pre-preparation protocol including treatment with TurboDNase buffer, TurboDNase, DNase I, and RNase cocktail (Invitrogen, Life Technologies, ThermoFisher Scientific, Waltham, MA, USA) was utilised before the extraction (paper II and IV).

Tissue samples, corresponding to approximately 1 cm^3, were cut from the frozen brain and the spinal cord (paper II) and cryolyzed using the Precellys tissue homogenizer (Bertin Corp. Rockville, MD, USA). RNA extractions from fluids (serum, saliva, urine, or semen) were performed without including a cryolyzing step (papers II and IV).

The RNA was then extracted by a trizolphenol-chloroform protocol and cleaned using the GeneJET RNA kit (ThermoFisher Scientific, Waltham, MA, USA) and included an "on-column" ezDNase™ Enzyme protocol to remove contaminating DNA (ThermoFisher Scientific, Waltham, MA, USA). Following the extraction, the RNA yields and quality, 260/280 ratio, were measured by a nanodrop spectrophotometer. When the extracted RNA was intended for high throughput sequencing, concentration was also recorded by a Qubit® 2.0 Fluorometer using the Qubit RNA HS assay kit (Thermo Fisher Scientific, Paisley, UK) and a TapeStation (Agilent, Santa Clara, CA, USA).

4.7 Synthesis of cDNA

The cDNA was synthesised from RNA originating from the brains of 15 piglets with signs of congenital tremor, the spinal cords of 13 piglets with signs of splay leg, and the brains of five healthy piglets (paper I). Three of the originally included eight healthy piglets had to be excluded from the study due to an insufficient RNA concentration. The RNA was treated with RQ1 RNAse-free DNAse (Promega, Madison, WI, USA) to remove potential contamination of genomic DNA. The cDNA was synthesised using a GoScript Reverse transcription system (Promega, Madison, WI, USA) (paper II) with an input of 1.2 µg RNA per reaction. As an extra control step for contamination of genomic DNA, a non-reverse transcribed control was run in parallel to the RNA samples, as described (Hjertner *et al.* 2013).

4.8 qRT-PCR (quantitative reverse transcription-PCR) for APPV

At present, no standardized methods to detect APPV are available. Moreover, the APPV genome is highly variable and may be difficult to detect by PCR (Sutton *et al.* 2022). Thus, two APPV-specific RT-qPCR assays targeting different parts of the genome were tested to improve the probability of detection: the NS3 encoding region and the NS5B encoding region. These two regions previously have been proven to produce reliable results (de Groof *et al.* 2016; Beer *et al.* 2017; Schwarz *et al.* 2017; Yuan *et al.* 2017; Cagatay *et al.* 2018; Mósena *et al.* 2018; Kaufmann *et al.* 2019; Sozzi *et al.* 2019; Folgueiras-González *et al.* 2020b; Grahofer *et al.* 2020; Riedel *et al.* 2021; Zhang *et al.* 2021; Arruda *et al.* 2022; Sutton *et al.* 2022; Yuan *et al.* 2022). Both assays were run under standard conditions on a Bio-Rad CFX96™ Real-time system in a C1000 Touch™ thermal cycler (Bio-Rad, Hercules, CA, USA). The assays generated comparable Cq values with an average standard error of ± 1.35.

The APPV-specific RT-qPCR protocol was based on the QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany) with a primer-pair targeting the NS3 encoding region of the APPV genome as described by Postel et al. (2016). One primer and one probe were slightly modified according to the only previous finding of Porcine pestivirus 1 (APPV) in Sweden (Blomstrom et al., 2016) to improve the chance of virus detection (paper I). A plasmid containing the NS3-encoding region of the APPV genome (kindly provided by Dr Alexander Postel at the University of Veterinary Medicine Hannover) was titrated to a concentration generating a constant Cq value of 25 and then used as a positive control.

Furthermore, the samples were tested using an APPV-specific RT-qPCR targeting the non-structural protein NS5B. This assay was based on the qScript XLT One-Step RT-qPCR ToughMix (Quanta Biosciences, Gaithersburg, USA) and ran as described by Beer et al. (2017). RNA from the APPV genome-positive piglet were confirmed APPV-genome positive

by the assay mentioned above with a Cq value of 26.5 and was used as a positive control (paper I).

The wild boar samples (Paper III) were sent to Germany for analysis at the University of Veterinary Medicine in Hannover (TiHo). The APPV-specific RT-qPCR protocol based on the QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany) and the primer pair and probe were used as described by Postel et al. (2016). Before running the APPV-specific qPCR, 119 serum pools were tested for PCR inhibitors by running a qPCR with the serum spiked with an artificial RNA. The samples were then pooled in pools of five wild boars and run in the APPV-specific qPCR. Of the total 119, 43 pools were PCR-positive. The wild boar sera of each APPV-positive pool were then tested individually by PCR.

4.9 Sanger sequencing

For downstream phylogenetic analysis, a part of the APPV NS3-gene was PCR-amplified and subjected to Sanger sequencing. Thus far, there is no consensus on which part of the APPV genome should be used for phylogenetic analysis, but the NS3 and NS5B coding regions, as well as the 5'-untranslated region (5' UTR) (Kaufmann *et al.* 2019; Sutton *et al.* 2022), have been suggested. The NS3 coding region was chosen since, at the time, the highest number of sequences were available from this region on GenBank and the European Nucleotide Archive.

In paper I, the APPV genome originating from one piglet per farm was Sanger-sequenced to enable phylogenetic analysis. Thus, amplicons from the APPV genome were generated from three piglets sampled in 2017/2018 in Västmanland, Blekinge, and Södermanland, and one piglet sampled in 2011 in Dalarna. Amplicons for Sanger sequencing were generated using the primers APPV_5087-fw and APPV_5703_Swe-rev (Stenberg *et al.* 2020a). Due to RNA fragmentation, no APPV amplicons could be obtained from the serum samples collected in 2004.

The NS3-encoding region of the APPV genome was also the focus of paper III. A melting-curve analysis of the APPV-positive samples indicated a

genetic diversity of the virus genome in fourteen serum samples. Of these, five sera originated from wild boars with metadata on at least the sampling year and the county and were selected for Sanger sequencing. Because the focus was on the phylogeny and comparison with APPV obtained from domestic pigs in Sweden, the selected samples included sera from Västmanland, Södermanland, and Blekinge, as APPV-sequences from these counties already were available. Amplification of an 806 bp long fragment within the NS3 encoding region was obtained using the primers APPV_5030-fw and APPV_5835-rev as described by Cagatay et al. (2018).

4.10 Phylogenetic analysis

For paper I, the phylogenetic analysis included 26 full and partial genome sequences mined from the GenBank, covering the NS3 encoding region. The sequences originated from domestic pigs and wild boars sampled in Germany, Austria, Spain, and China. No cluster approach was applied to the tree as it comprised only 30 APPV sequences.

The trees were estimated using the MAFFT alignment tool and the PHYLIP Neighbor-Joining method in the UGENE software (Okonechnikov *et al.* 2012). The bootstrap value was set to 1000. For comparison, Bayesian trees were estimated using the MR Bayes tool within the UGENE software. The Bayesian trees and the Neighbor-Joining trees were consistent with each other.

For papers III and IV, Dr Michelle Wille at the University of Sydney kindly provided us with more comprehensive trees. The tree presented in paper III, included all APPV NS3 sequences available in GenBank at the time, including the five NS3 sequences obtained from the Swedish wild boars. The sequences were aligned using the aligner MUSCLE (Edgar 2004) in the Genious Prime software (https://www.geneious.com). Maximum likelihood trees were estimated by implementing the best nucleotide substitution model in PhyML (Guindon et al., 2010). Support values were calculated using aBayes, a Bayesian approach for rapid support estimation (Anisimova *et al.* 2011).

The trees for paper IV included an APPV tree and a tree of the Posavirus -1 contigs detected in the semen. For the trees, all available Posavirus-1 and APPV sequences were mined from GenBank and aligned using MAFFT and the E-INS-i algorithm, implemented in the Geneious prime software (https://www.geneious.com). As the recovered contigs spanned different parts of the genome, an approach of removing the regions of the alignments between contig positives was taken. To capture all three Posavirus contigs, three alignment blocks ranging from position 1072-3987 (non-structural region spanning the RNA helicase) relative to NC_023637 were concatenated, for a final alignment length of 1446bp. To capture both APPV contigs, the 5'UTR and a region spanning the end of Erns and the first half of E1, were concatenated for a final alignment length of 692bp. Maximum likelihood trees were estimated using PhyML, implementing the best nucleotide substitution model as determined by "smart model selection", integrated into PhyML (Guindon et al., 2010). Support values were calculated using aBayes (Anisimova et al., 2011).

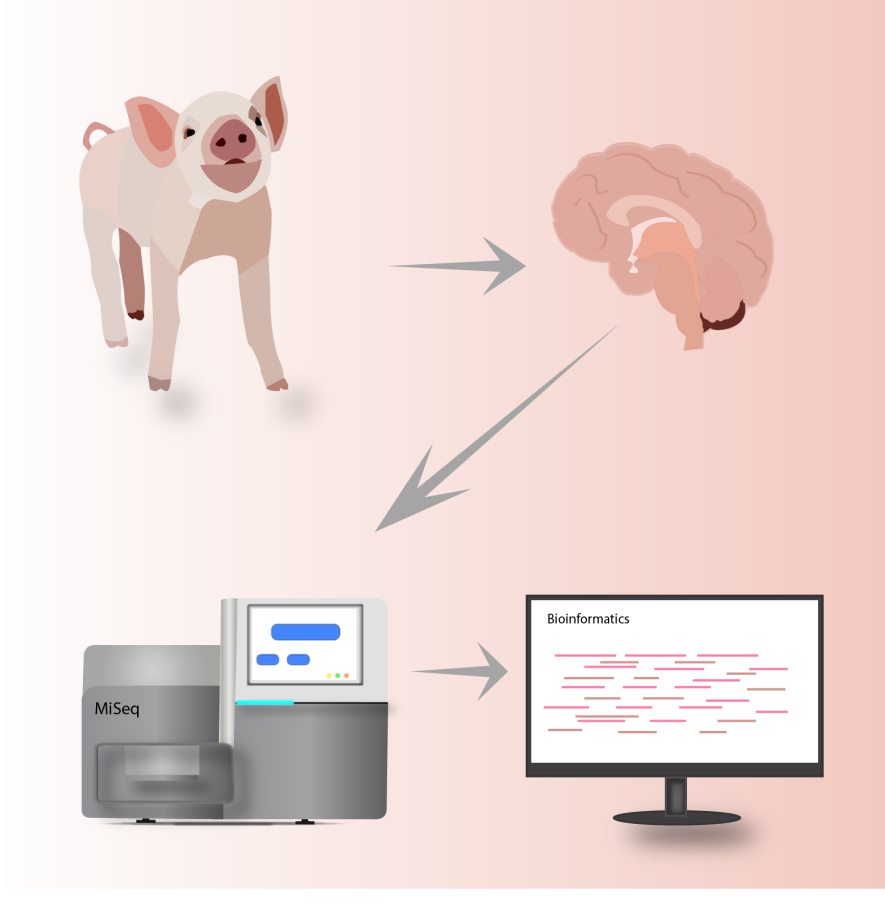
4.11 High-throughput sequencing

The sequencing was performed in batches on two Illumina platforms (MiSeq and NovaSeq) with different throughputs at the SNP & SEQ Technology Platform in Uppsala. The samples were sequenced two times, with an increased sequencing depth the second time for better detection of viral reads. In this thesis, the RNA virome was the focus.

From January to March 2020, three sequencing runs were performed: first, brain tissue from the eight healthy piglets and thereafter spinal cord tissue from six piglets with signs of the splay leg. Finally, eight semen pools were subjected to sequencing. The sequencing was a paired-end 75 bp read length sequencing using a MiSeq Reagent Kit v3, 600 cycles on a MiSeq instrument. A sequencing library for the phage PhiX was included as a 5% spike-in into the sequencing run. None or a few viral reads were detected in the datasets generated by the MiSeq sequencing. Therefore, a sequencing run with a higher throughput was performed in June 2020: a paired-end 150 bp read length sequencing using an SP flow cell and the v1 sequencing chemistry on the NovaSeq 6000 system. A 1% spike-in with a sequencing

library for the phage PhiX was included in the run. A total of 30 samples were included in this run: brain tissue from eight healthy piglets, spinal cord tissue from six piglets with signs of splay leg, eight semen pools, and brain tissue from eight piglets with signs of congenital tremor whereof, six were PCR-positive for APPV and two were PCR-negative for APPV. The quality of the datasets was assessed using the FastQC (Andrews 2010).

Figure 7. Schematic flow chart of the high through-put sequencing process



4.12 Miseq datasets bioinformatics analysis

The bioinformatics analysis of the datasets produced by MiSeq sequencing included the following steps: quality control and trimming of reads by FASTP version 0.19.5 (Chen *et al.* 2018b) and removal of host reads by using bowtie2 version 2.4.1 (Langmead & Salzberg 2012) to map against the *Sus scrofa domesticus* genome. Further, *de novo* assembly of the reads to contigs was done using MEGAHIT version 1.2. 9 (Li *et al.* 2015). The reads and contigs were then analysed using three different classifiers for the output comparison: Kaiju Version 1.7.3 (Menzel *et al.* 2016), DIAMOND v0.9.31 (Buchfink *et al.* 2015), and Kraken2 version 2.0.8-beta (Wood *et al.* 2019). Kaiju and Kraken2 are the two preferred taxonomic classifiers for metagenomic analysis when focusing on viruses (Carbo *et al.* 2022). Kaiju was run on the reads and the contigs against the non-redundant protein (*nr*) NCBI database. It classified <0,1 % of the reads as viral and detected no viral contigs. Kraken2 version 2.0.8-beta was run on both the reads and the contigs using the non-redundant protein database (*nr*) and the non-redundant nucleotide (*nt*) NCBI database. DIAMOND was run on reads and contigs against the non-redundant protein database (*nr*).

Additionally, specific alignments against the APPV genome (NC_030653.1 Atypical porcine pestivirus 1 isolate Bavaria S5/9 polyprotein gene, complete cds) were performed using Bowtie2 version 2.3.5.1 (Langmead & Salzberg 2012).

4.13 NovaSeq datasets bioinformatics analysis

For analysing the datasets generated by NovaSeq, a Nextflow pipeline was developed, taking the results of the bioinformatical analysis of the MiSeq data into account. The pipeline is fully available online at GitHub (https://github.com/jhayer/nf-metavir). The following steps were included: quality control and trimming of the reads using FASTP version 0.19.5 (Chen *et al.* 2018b), removal of the host reads by mapping on *Sus scrofa domesticus* genome using bowtie2 version 2.3.5.1 (Langmead & Salzberg 2012), and *de novo* assembly of the remaining reads using MEGAHIT version 1.2.9 (Li *et al.* 2015). For comparison, the pipeline was run with and without host mapping. Taxonomic classification of the reads

was performed using Kraken 2 version 2.0.8-beta (Wood et al., 2019) against the nucleotide non-redundant (*nt*) NCBI database. For the assembled contigs, two methods were used and compared for taxonomic classification: Kraken2 and DIAMOND version 0.9.24.125 (Buchfink *et al.* 2015). Kraken2 was run against the non-redundant protein database (*nr*) and the non-redundant nucleotide (*nt*) NCBI database, and DIAMOND was run against the *nr* protein database. Kaiju Version 1.7.3 (Menzel *et al.* 2016) was also run on the reads and the assembled contigs of the non-redundant protein (*nr*) NCBI database. Interestingly, Kraken 2 was the only classifier that could identify reads and contigs as APPV in samples that were PCR-positive for APPV-genome. The resulting reports were visualised using Pavian (Breitwieser & Salzberg 2020). Additionally, specific alignments against the APPV genome (NC_030653.1 Atypical porcine pestivirus 1 isolate Bavaria S5/9 polyprotein gene, complete cds) were performed using Bowtie2 version 2.3.5.1 (Langmead & Salzberg 2012).

4.14 Assay of RT-qPCR

In this study, the focus was to evaluate the expression of a panel of genes in the grey matter of the brain from piglets suffering from natural infection with APPV (paper II). Hence, the gene expression in the brain of the piglets with signs of congenital tremor and being PCR-positive for APPV (n =13) were related to the gene expression of five healthy piglets, PCR-negative for APPV.

For this purpose, a panel of porcine genes were selected to represent pro-inflammatory signals (IL-1β, IL-6, CXCL8, IFN-γ), downregulatory signals (IL-10), and genes related to an immediate anti-viral response (IFN-a, IFN-β, IFITM3, STING). The gene expression was assessed using previously published and optimised primer pairs under assay-specific conditions (Ahlberg *et al.* 2017). For comparison, cytokine genes upregulated in the APPV-infected piglets were analysed in the spinal cord tissue from piglets with signs of splay leg (n = 12) and in the brain tissue from the two APPV-negative piglets displaying signs of congenital tremor.

4.14.1 Reference genes and normalization of gene expression

To enable normalisation of the data, primer pairs for five reference genes (GAPDH, HPRT, PPIA, RPL32, and YWHAZ) were tested for their expression stability in the grey matter of porcine brain tissue under established assay conditions (Ahlberg *et al.* 2017; Hjertner *et al.* 2021). Using the geNorm software (qBasePLUS, Biogazelle) to evaluate the gene stability parameter (M) and coefficient of variation (CV), three genes (GAPDH, HPRT, and RPL32) were selected for the normalisation of gene expression (paper II).

4.15 Enzyme-linked immunosorbent assay (ELISA)

The wild boar samples (Paper III) were sent to Hannover, to the University of Veterinary Medicine, for analysis using their in-house APPV-specific enzyme-linked immunosorbent assay (ELISA). This is an indirect ELISA, based on the APPV glycoprotein E^{rns} for antibody detection (Postel *et al.* 2017b). The reliability of the ELISA was assessed by first analysing 88 randomly selected serum samples, proportionally chosen from each year, in duplicates. Once the reliability was established, the remaining serum samples were analysed as singletons.

The results from the ELISA study are presented as 'S/P-values' (sample absorbance values/positive control) to ensure reliable inter-assay comparability and enable inter-study comparability. For the inter-study comparability, the S/P-values' were also classified into low ($S/P \leq 0.5$), intermediate ($0.5 < S/P < 1.0$), or high serum reactivity ($S/P \geq 1.0$) in accordance with other studies (Postel *et al.* 2017b; Cagatay *et al.* 2018; Grahofer *et al.* 2020). Since the ELISA used for this study was used by Postel et al. (2017), Cagaty et al. (2018), and Grahofer et al. (2020) and in the same laboratory setting, the same cut-off value, $S/P = 0.5$, was applied to the present study. Accordingly, wild boars with a serum reactivity (S/P) ≤ 0.5 were regarded as seronegative and wild boars with a serum reactivity (S/P) > 0.5 as seropositive.

4.16 Statistical analysis

The statistical analyses for paper II were conducted using the Prism 7.0 (Graph-Pad) and JMP® Pro 15.2.0 software. An unpaired t-test was used on the ΔΔCq values to test for differences in the expression of cytokine genes of the APPV-negative healthy piglets and the APPV-positive piglets suffering from congenital tremor. Then, a bivariate fit analysis was used to test for correlation in the expression of STING and IFN-α. The model included the thirteen observations of the STING and IFN-α expression from APPV-genome positive piglets with signs of congenital tremor. For all tests, p-values below 0.05 were regarded as significant.

The statistical analyses for paper III were performed using the JMP® Pro 15.2.0 software. The wild boar statistics were reported as means and percentages. The dataset was analysed for differences in antibody reactivity between wild boar gilts and sows, annual differences, seasonality, and differences between the counties. A general linear model that included the sampling year, county, and season as fixed factors, was implemented to analyse the serum reactivity (S/P-value). This model included 395 of the total 595 observations and excluded 200 due to insufficient metadata. To study how the reproductive status (gilt/sow) affected the serum reactivity, a general linear model, only including observation from gilts and sows, was set up. The model included 116 of the 595 wild boar observations, but these 116 observations originated from only four counties (Blekinge n = 35, Skåne n = 23, Södermanland n = 57, and Uppland n = 1) and two years (2013–2014).

Tukey's honest significant difference test, the significance level set to $\alpha = 0.05$, was used for pairwise comparison of each factor (year, county, and season). Since the APPV-genome-positive samples only comprised 73 wild boars, no statistical tests were performed on the PCR data.

5. Results and discussion

5.1 Detection of atypical porcine pestivirus in the brain tissue of piglets suffering from congenital tremor type A-II (Paper I)

Twenty-nine piglets with signs of CT type A-II were tested for APPV using RT-qPCR assays targeting two distinct regions of the APPV genome. The piglets originated from seven farms and were sampled in 2004, 2012, and 2017/2018. The collected material consisted of serum samples (n = 11, sampled 2004) and brain tissue samples (n = 3, sampled 2012 and n = 14, sampled 2017/2018).

Of the tested piglets, 27 of the total 29 were PCR-positive for APPV, whereas two piglets from the same farm and litter, sampled in the year 2018, were PCR-negative for APPV. The results from this study were the first detection of APPV in Swedish piglets with signs of CT type A-II. However, a porcine pestivirus with a 90% nucleotide similarity to the APPV described by Hause et al. (2015) was detected by high throughput sequencing in a lymph node from a Swedish piglet with signs of PMWS in the year 2016 (Blomstrom *et al.* 2016). The pestivirus was not associated with disease and was mentioned only as an incidental finding.

Thus, the present thesis establishes that APPV has circulated in Sweden since at least the year 2004. However, since there are reports of CT in Sweden from 1955 (Larsson 1955), it may be possible that APPV has been present in the Swedish pig population for several decades.

5.2 Detection of atypical porcine pestivirus in the urine and saliva of piglets suffering from congenital tremor type A-II (Paper I)

In addition to brain tissue, urine and saliva were sampled from five piglets with signs of CT type A-II. All five were littermates and PCR-positive for APPV-genome in the brain tissue and saliva. Four out of five piglets were also PCR-positive for APPV-genome in urine.

The detection of APPV in saliva is in line with the findings of others (Postel *et al.* 2016; Schwarz *et al.* 2017; Arruda *et al.* 2022) and in line with previous knowledge of the excretion of pestiviruses (Meyling *et al.* 1990; Choi & Chae 2002). The detection of APPV in saliva opens the possibility of sampling methods other than conventional blood sampling as a recent study suggests that oral fluid (saliva) is at least as reliable as serum for the detection of APPV (Arruda *et al.* 2022). Further, both Arruda et al. (2022) and Schwarz et al. (2017) reported higher viral loads in oral swabs compared with serum or nasal swabs (Schwarz *et al.* 2017; Arruda *et al.* 2022). Reliable APPV detection from saliva is an important discovery since sampling saliva is less invasive, more animal-friendly, and quicker to perform compared to blood sampling.

5.3 No detection of atypical porcine pestivirus in the spinal cord tissue of piglets suffering from splay leg (Paper I)

Thirteen piglets with signs of splay leg were sampled in 2017, and included in this study. The piglets originated from four farms and six litters. None of the tested piglets was PCR-positive for the APPV genome in the spinal cord tissue, despite using two RT-qPCR assays targeting distinct regions of the APPV-genome.

In the literature, there are reports of co-morbidity between CT type A-II and splay leg following infection with APPV (Arruda *et al.* 2016; de Groof *et al.* 2016; Schwarz *et al.* 2017; Gatto *et al.* 2018b; Houston *et al.* 2022). Thus, APPV has by some been suggested as one causative agent of splay leg, but the causality between APPV and splay leg is to this date not established

(Arruda *et al.* 2016; de Groof *et al.* 2016). However, given the distinct epidemiological pattern of naturally occurring cases of splay leg and CT type A-II and the absence of reports of APPV-positive piglets with splay leg as the single clinical sign, it seems unlikely that APPV is the causative agent for splay leg.

5.4 Pathology

5.4.1 Post mortem examinations (paper I & II)

Gross lesions associated with the disease were absent in all piglets. The healthy control piglets and the piglets with signs of congenital tremor all had suckled, and there was coagulated milk in their ventricles and faeces in their intestines. Piglets with signs of splay leg had empty ventricles and intestines, and, thus, had not suckled.

These findings agree with the literature. Most piglets suffering from CT type A-II manage to nurse and recover from the syndrome within a few weeks or months (Arruda *et al.* 2016; de Groof *et al.* 2016; Schwarz *et al.* 2017; Cagatay *et al.* 2019). In theory, splay leg is also a transient syndrome. Nevertheless, the lethality is higher compared to CT type A-II. The lethality of splay leg could reach 50% mainly due to starvation if appropriate care *e.g.,* supplementary feeding, is not given (Zelená & Jirmanová 1979; Ooi *et al.* 2006; Schumacher *et al.* 2021).

5.4.2 Histopathological examinations of the brain and spinal cord of piglets with signs of CT type A-II or splay leg

Hematoxylin and Eosin stain (paper II)

Vacuoles were present in equal numbers in the brain and spinal cord of healthy piglets and piglets with signs of congenital tremor or splay leg. A few small and multifocal vacuoles with varying shapes and sizes were observed in the brain's white brain, and at all levels of the spinal cord. The vacuoles were scattered in the tissue and did not display any associated cell reactions. No debris was seen within the vacuoles. In the brain, the vacuoles

were most abundant in the cerebellum, but they common in the spinal cord than in the brain.

As described, vacuoles were seen in equally small numbers in healthy piglets and piglets with signs of CT type A-II or splay leg. Thus, these vacuoles were considered normal findings. The absence of lesions agrees with Zhang et al. (2017) but disagrees with others who described more severe lesions (Mósena *et al.* 2018; Possatti *et al.* 2018a; Possatti *et al.* 2018b; Ren *et al.* 2022).

So far, five A-types and one B-type of congenital tremor have been established (Done 1968; Patterson *et al.* 1976; Done *et al.* 1986; Done & Paton 1995). A-types are characterised by hypomyelination and vacuolisation in the brain's white matter, whereas the B-type is not associated with any microscopical lesions and has no known causative agent. Additionally, the epidemiology of the B-type is distinct from the A-types (Done 1968; Patterson *et al.* 1976; Done *et al.* 1986; Done & Paton 1995). As the Swedish piglets with signs of congenital tremor lacked microscopical lesions, it may be argued that they suffered from congenital tremor type B. However, all of them, including the two APPV-negative piglets, originated from litters where all piglets displayed signs of congenital tremor, an epidemiological pattern consistent with CT type A-II.

Since the establishment of the six types of congenital tremor, most publications on CT type A-II include an incumbent description of vacuoles within the brain's white matter, ranging from subtle to severe. However, many studies lack information on the time between death and tissue fixation. The study describing the most severe lesions included four piglets found dead on the farm and transported before being subjected to a post-mortem examination (Possatti *et al.* 2018a). Vacuoles in the central nervous system are common artefacts due to autolysis and delayed fixation (Vandevelde 2012; Wohlsein *et al.* 2013). Such artefact vacuoles are often particularly prominent within the white matter. Further, vacuolisation may also occur due to infection with numerous other viral agents than APPV, and sporadic vacuoles may be present in the healthy brain. Thus, prompt fixation of tissue post-mortem, control of co-infections, and healthy controls are crucial when evaluating and associating lesions with an infectious agent. As knowledge of

CT type A-II and APPV expands, it may be speculated that a slightly more differentiated description of the histological lesions associated with the different types of congenital tremor will be required.

Luxol fast blue stain and cresyl violet stain (paper II)

The general hypothesis of the pathogenesis behind congenital tremor is that the tremor occurs due to loss of myelin. The decrease of myelin can be studied by specific stains such as Luxol fast blue. Further, the neurones of the CNS can be studied by an additional staining, cresyl violet stain. Thus, myelin loss and/or loss of Nissl substance of the cerebellum and spinal cord of the piglets were studied using Luxol fast blue and cresyl violet stain in combination. The study included four piglets: one healthy and three APPV-positive piglets with signs of congenital tremor. No pathological findings consistent with alterations, or loss of Nissl substance or a decreased amount of myelin could be detected.

The absence of myelin loss is in contrast to other studies on piglets with signs of congenital tremor and PCR-positive for APPV that describe a mild to moderate hypomyelination of the spinal cord (Postel *et al.* 2016; Schwarz *et al.* 2017; Dessureault *et al.* 2018; Possatti *et al.* 2018b; Ren *et al.* 2022) and of the cerebellum (Schwarz *et al.* 2017). Further, one study by Possatti et al. (2018) that used cresyl violet stain reported severe changes to the neuronal cell bodies of the CNS. However, this study is not fully comparable to ours because it was conducted on piglets found dead on the farm and included no matched healthy controls. Therefore, the described changes seen in neuronal cell bodies and attributed to the APPV infection (Possatti *et al.* 2018b) cannot be clearly differentiated from post-mortem changes.

Staining with Congo Red (in addendum)

Amyloid is a protein aggregation associated with alterations of myelin, such as vacuolisation and neurological diseases in humans, *e.g.*, Parkinson's disease, Alzheimer's disease, and Creutzfeldt–Jakob disease. Recent research has suggested that viral presence in the brain may catalyse aggregation of the amyloid β-peptide that induces myelin vacuolisation (Ezzat *et al.* 2019; Malmberg *et al.* 2020). The presence of amyloid plaques in piglets with signs of congenital tremor was investigated since the vacuolisation of the brain's white matter brain described in association with

APPV to some extent is similar to that observed in degenerative amyloid-associated diseases.

Two APPV-positive piglets with signs of congenital tremor and one healthy piglet were investigated for amyloid plaques in the brain and spinal cord. However, Congo Red staining could not reveal amyloid aggregation in the tissues.

Transmission electron microscopy (paper II)

As no microscopical lesions were seen in the CNS of piglets with signs of congenital tremor, a transmission electron microscopy study was performed to investigate ultrastructural lesions that could begin to explain the clinical signs. Thus, tissue from the cerebellum from two piglets was examined via transmission electron microscopy: one from a healthy piglet (PCR-negative for APPV-genome) and one from a piglet with congenital tremor (PCR-positive for APPV-genome). Minor ultrastructural changes, such as mild separation of myelin lamellae, were seen in the cerebellar tissue of the clinically unaffected piglet. Mild separation of myelin lamellae was also seen in the piglet with signs of CT type A-II, in addition to degenerated mitochondria and small sporadic vacuoles of the neuropil. The degenerated mitochondria, characterized by swelling and loss of crista structure, were mainly present in the oligodendrocytes but occasionally in the axons.

The piglet with signs of CT type A-II showed mild ultrastructural changes comparable to the findings by Schwarz et al. (2017). Mild splitting of myelin sheaths, as seen in the healthy piglet and the piglet with signs of CT type A-II, is a common artefact seen in the central nervous system, especially in the thicker myelin sheaths. Thus, the splitting of myelin sheaths cannot be attributed to infection with APPV.

The only evident lesion in the cerebellum of the piglet with signs of CT type A-II were the degenerated mitochondria primarily seen in the oligodendrocytes, the oligodendrocyte precursor cells. The oligodendrocytes are the myelin-forming cells of the brain, and the myelin sheets provide metabolic support, insulate underlying axons, and are needed for the saltatory signal transduction of neuronal action potentials (Nave 2010). An injury or death of an oligodendrocyte can lead to failure of neuronal

signalling (Rosko *et al.* 2019), which may result in tremor (Gow & Devaux 2008) or other neuronal signs.

Figure 8. TEM image of the cerebellum

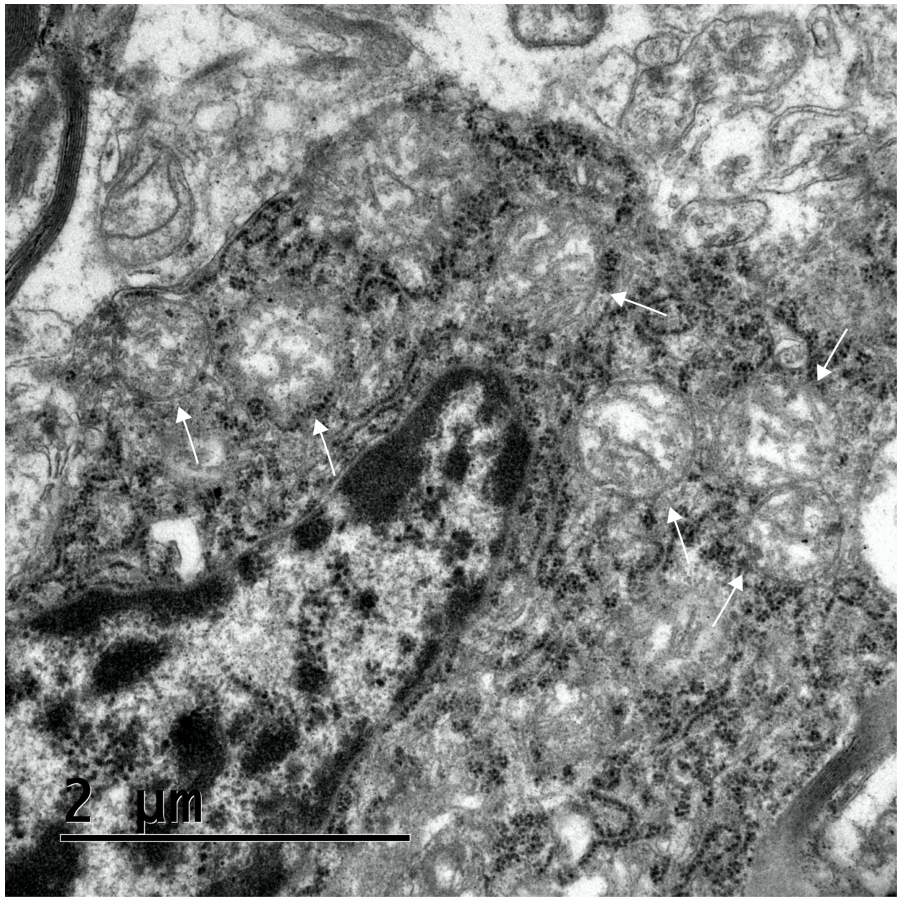


Figure 8. TEM image of the cerebellum. The degenerated mitochondria, characterized by swelling and loss of crista structure, are marked with white arrows.

Oligodendrocytes have a high metabolic demand for mitochondrial-derived ATP, especially during the myelination process (Meyer & Rinholm 2021). Throughout the oligodendrocyte development, the mitochondria change in distribution and morphology, and once the process of myelination is

complete, the oligodendrocytes require less intense mitochondrial respiration (Meyer & Rinholm 2021). In the myelin sheets, mitochondria are fewer and smaller as compared to mitochondria in oligodendrocyte precursor cells. These mitochondria show higher resilience towards oxidative stress. Oligodendrocyte precursor cells and their mitochondria, however, are sensitive to oxidative stress (Thorburne & Juurlink 1996; Kim *et al.* 2020), and they may show morphological anomalies when their high metabolic demand is not met (Zhou *et al.* 2018). This implies that the growing brain is more sensitive to stress, such as infections than the adult brain.

Mitochondrial degeneration of the oligodendrocyte precursor cells, as observed in the piglet with signs of CT type A-II, suggests that the cells have been subjected to stress, *e.g.*, a viral infection. This degeneration also indicates a negatively affected myelination. It is unclear whether these subtle changes are severe enough to induce tremor, but they suggest that APPV infection interferes with the myelin-producing cells.

5.5 The cytokine profile of APPV-infected brains (paper II)

The APPV- infection and immunity, with persistent carriers and congenital disease, is complex and not fully characterised. Thus far, the present study is the only description of the local immune response of the brain in association with APPV infection.

The expression of cytokine genes CXCL8, IFN−α, IFN-β, IFN-γ, IL-1β, IL-6, IL-10, and STING (Stimulator of interferon genes) in association with infection with APPV, was studied in brain tissue from 13 piglets, PCR-positive for APPV-genome and with signs of congenital tremor, and compared with the cytokine gene expression in samples from eight clinically healthy piglets.

When comparing the gene expression in brain tissue of healthy piglets with the gene expression of APPV-infected piglets, the gene encoding STING was found to be significantly up-regulated ($p < 0.05$) (using an unpaired t-test on the ΔΔCq values). Further, an upregulation of the gene encoding IFN-α was indicated, although not statistically significant. Nevertheless, a

bivariate analysis found a significant correlation between the expression of STING and IFN-α ($r = 0.824$, $p < 0.0001$).

The gene encoding IFITM3 was detected in all brain samples, but not differentially expressed in the piglets suffering from CT (FC: 1.1 ± 1.9) compared with the healthy control group (FC: 1.4 ± 1.5). The expression of the genes encoding CXCL8, IFN-β, IFN-γ, IL-1β, IL-6, and IL-10 was not detected in the brain tissue of APPV-positive piglets. Nevertheless, these genes were detected, although at high Cq values, in the brain tissue of the healthy piglets.

The cytokine genes that were detected in the brain of the APPV-infected piglets (STING, IFN-α, and IFITM3) were also analysed in the spinal cord tissue from piglets with signs of splay leg ($n = 12$) and in the brain tissue from two APPV-negative piglets displaying clinical signs of congenital tremor. No expression of the selected genes was found in these animals.

APPV appears to induce a STING-associated cytokine profile in porcine brain tissue. Supporting this, the genes encoding STING and IFN-α were not differentially expressed in the brain tissue from the two piglets with signs of congenital tremor that were negative for the APPV-genome.

STING (Stimulator of interferon genes) is a protein involved in the activation of type I interferons (Hopfner & Hornung 2020). The production of these interferons is mainly mediated through interferon regulatory factor 3 (IRF3) and interferon regulatory factor 7 (IRF7)-dependent signalling (Honda *et al.* 2006; Suschak *et al.* 2016). IRF3 and IRF7 are the principal mediators of IFN-β and IFN-α, respectively (Wu & Chen 2014). The central function of these cytokines is to induce an antiviral cell stage and to direct the antiviral immune response. Further, the absence of cytokines in the APPV-infected brain tissue supports the proposed regulatory effect of the STING pathway to protect the brain from damage from high levels of type-I IFNs (Reinert *et al.* 2021). However, the low occurrence of cytokines in the APPV-infected brain may also be attributed to the suggested immunoregulatory potential of APPV (Mou *et al.* 2021; de Martin & Schweizer 2022). The immunoregulatory effect as interferon antagonists of the N^{pro} and E^{rns} proteins encoded in the APPV genome is well described for other

pestiviruses (Tautz *et al.* 2015). The N^{pro} inhibits the production of the IRF3 (Randall & Goodbourn 2008; Liu *et al.* 2015), which leads to decreased downstream production of IFN-β (Honda *et al.* 2006). Similarly, the E^{rns} protein inhibits the production of PAMPs (pathogen-associated molecular patterns), leading to a decrease in the stimulation of INF-genes genes (Kawasaki & Kawai 2014).

Despite the indicated immunoregulatory traits of APPV, an appropriate antiviral immune reaction that clears the APPV infection will be elicited in most piglets (Cagatay *et al.* 2019). To better understand the immune response in the brain during APPV infection, a comprehensive analysis is necessary, for example, transcriptome sequencing. Additional studies are also required to understand the suggested immunoregulatory potential of APPV and the systemic immune reactions in the APPV-infected pig.

5.6 Metagenomic sequencing

5.6.1 Metagenomic sequencing of CNS-tissue (paper II)

The samples included in this study were first sequenced on an Illumina MiSeq platform and thereafter on an Illumina NovaSeq platform. A mistake was made when writing paper II, and all described results concerning the sequencing included in the paper came from sequencing on an Illumina NovaSeq platform.

In the datasets generated from the MiSeq sequencing, only a few reads classified as viral were recovered. As the brain is considered to have very limited viral richness (Pyöriä *et al.* 2023), a low number of viral reads was expected. However, due to insufficient sequencing depth, no reads classified as APPV were recovered, despite the APPV genome being detectable by qPCR. Consequently, a new sequencing run with increased sequencing depth was required. Following a discussion with the sequencing coordinators at SciLifeLab, the sequencing libraries were re-run on the NovaSeq platform for greater sequencing depth. The NovaSeq produced datasets where reads taxonomically classified as APPV could be recovered. Thus, the

results were deemed more reliable compared with the results generated from the MiSeq platform.

The sequencing output from the NovaSeq run is presented in the table in paper II. Over all samples, between 80-90% of the reads mapped against the *Sus scrofa domesticus*-genome and less than 1% of the reads were classified as viral. However, most of the reads classified as viral were not relevant hits but were misclassifications of the host-derived reads or contaminants from reagents and humans. It is difficult to eliminate host-derived reads from a dataset, which leads to a positive bias in the taxonomical classification of microorganisms (Lee *et al.* 2016). In addition, most classifiers also suffer from false identification of microorganisms seen in the output as an abundance of false positive hits at a low read count (Ye *et al.* 2019). For Kraken 2, this is particularly common if the material is host-rich (Lee *et al.* 2016; Wood *et al.* 2019). There is also a known problem with contaminating sequences in all datasets, particularly in datasets derived from material with low microbial biomass (Eisenhofer *et al.* 2019), such as the CNS. Therefore, it is crucial to perform a careful bioinformatic analysis and exercise caution when interpreting results from all classifiers: the databases contain genomes that may be wrongly annotated or taxonomically subdivided into distinct species or genera, which could share a high genetic identity.

All laboratory reagents come with a set of contaminating sequences, a so-called "kitome" (Asplund *et al.* 2019). Thus, contaminating reads could be detected in all datasets, including the negative RNA-control: *e.g.*, reads classified as Hepatitis E virus, Human immunodeficiency virus, and Influenza A virus. Most contaminating reads followed a pattern described by others (Asplund *et al.* 2019; Ye *et al.* 2019): low in numbers and with a stochastic appearance over the datasets. However, reads classified as Equine infectious anemia virus were recovered from all datasets, including the one generated from the negative RNA-control. These reads probably originate from a reagent contaminant, a novel reagent-associated lenti-like virus, misclassified as Equine infectious anemia virus (Porter *et al.* 2021). Further, Human respovirus-1 reads were detected in the datasets from one sequencing batch. Human respovirus-1 is excreted through the upper airways and causes the common cold in humans. This virus was probably introduced into the samples when preparing the sequencing libraries. Viral contaminants derived

from the pig genome were also commonly recovered. For example, reads classified as Porcine type-C oncovirus, an endogenous retrovirus integrated into the genome of all pigs (Denner 2017; Denner 2021). Compared to the bacterial and fungal parts of the microbiome, the virome has proven more challenging to describe (Wang 2020). Further, the occurrence of both DNA and RNA viruses has to be taken into account if the full virome is to be described. In this thesis, however, the focus was on the RNA part of the virome.

Despite rapid progress in sequencing methodologies and bioinformatics analysis, the sampling methods and library preparations will affect what can be recovered from the datasets. In addition, all datasets will hold a viral "dark matter" (Santiago-Rodriguez & Hollister 2022). Thus, with the present methods at hand, it is reasonable to assume that the full virome of any given niche cannot be comprehended. For example, in opposite to bacteria, viral genomes do not have conserved marker sequences. This lack of marker sequences requires an increased sequencing depth and more bioinformatic analysis compared with 16S rRNA sequencing of bacteria. Accordingly, comprehensive sequencing was done for the studies included in this thesis, and different approaches, classifiers and databases were applied to the bioinformatics analysis. By using both "RNA to RNA methods" and "RNA to protein methods", a high sequence specificity and sensitivity to the detection of novel sequences has been obtained (Asplund *et al.* 2019; Ye *et al.* 2019).

NovaSeq sequencing of brain tissue from healthy piglets (paper II)

Metagenomic sequencing was performed on brain tissue obtained from eight clinically healthy piglets, all PCR-negative for the APPV-genome. After quality control, filtering, and removal of the host genome, about 50% of the remaining reads were of microbial origin. Less than 0.1% of the reads were classified as viral. Reads classified as Porcine type-C oncovirus were recovered in all datasets. Alignment against the APPV-genome did not generate any hits. A few viral contigs were detected in all datasets but were misclassifications derived from the *Sus scrofa domesticus* genome.

NovaSeq sequencing of spinal cord tissue from piglets with signs of splay leg (paper II)

The spinal cord tissue from seven piglets with signs of splay leg, PCR negative for APPV-genome, was subjected to sequencing on a NovaSeq platform. Six libraries were sequenced together as one batch, and one library was sequenced with the libraries originating from piglets with signs of congenital tremor. After quality control, filtering, and removal of the host genome, about 45% of the remaining reads were of microbial origin. Less than 0.1% of the reads were classified as viral. Alignment against the APPV-genome did not generate any hits, and there was no detection of valid viral reads in any dataset. In each dataset, several reads and contigs classified as bacterial were discovered. However, upon extraction and BLAST analysis, they aligned with the *Sus scrofa domesticus* genome.

NovaSeq sequencing of brain tissue from the piglets with signs of congenital tremor (paper II)

Samples from brain tissue originating from six piglets with signs of congenital tremor sampled at four farms in 2017/2018 were sequenced. Four piglets included in the study were PCR-positive for the APPV-genome in the brain tissue, and two were PCR-negative for the APPV-genome.

After quality control, filtering, and removal of the host genome, about 6% of the remaining reads were of microbial origin. Overall datasets, less than 1% of the reads were classified as viral. Reads classified as APPV-genome could be recovered from the datasets derived from the APPV PCR-positive piglets. Specific alignment against the APPV-genome also identified APPV sequences in the datasets from the four PCR-positive piglets, but not in datasets from the two PCR-negative piglets. In all datasets, reads classified as APPV were the most abundant viral reads, generating 140 reads at most in one dataset.

Of the viruses generating > 10 reads in at least one dataset, only APPV, Porcine type-C oncovirus, and Aichivirus C were valid viral hits. Reads classified as Porcine type-C oncovirus were recovered from all datasets. Reads classified as APPV were present in the dataset of four out of six piglets, and reads classified as Aichivirus C were present in three datasets from piglets with signs of congenital tremor infected with APPV. Of these three viruses, only APPV generated contigs.

Porcine type-C oncovirus was the only virus that generated BLAST-validated reads in the datasets from the piglets that were PCR-negative for APPV-genome but with signs of congenital tremor. No viral contigs could be recovered from the datasets derived from these two piglets.

Regardless of the piglets' health status, only a few viral reads and contigs were recovered over all datasets. Reads classified as APPV were the most numerous of the valid viral hits. Nevertheless, these reads were present in much lower numbers than reads from the novel reagent-associated lenti-like virus. Although reads derived from the APPV genome were detected in the datasets from all APPV PCR-positive piglets, the low number of APPV reads indicates that other viruses of less abundance could go undetected.

In addition to the detection of APPV, a few reads classified as Aichivirus C were recovered in three datasets originating from three APPV-positive piglets with signs of CT type A-II and from different farms. Reads classified as Aichivirus C have been recovered in other sequencing studies (Eriksen 2023) and the virus was loosely associated with neonatal diarrhoea in piglets (Theuns *et al.* 2018). However, a recent systematic review on the topic has established that Aicivirus C is of low clinical relevance and probably an incidental finding in pigs (Eriksen 2023).

The low recovery of viral reads is a known difficulty when performing high-throughput sequencing on RNA. According to Pyöriä et al. (2023), viral RNA treated for metagenomic sequencing is less stable and degrades more easily compared to DNA. Thus, there is an overall higher agreement between high-throughput sequencing and qPCR when working with DNA compared to RNA (Pyöriä *et al.* 2023).

Based on the sequencing results, it can be speculated that the complete tissue resident CNS RNA-virome of piglets is sparse. A sparse tissue resident CNS virome, as recovered in the piglets, is in line with the current literature from human studies (Ghose *et al.* 2019; Kumata *et al.* 2020; Yuan *et al.* 2020; Link 2021; Pyöriä *et al.* 2023). Nevertheless, the complete tissue resident CNS microbiome, including the virome, is yet not characterised for any

species, and it may be assumed that in the future, with better methods of higher sensitivity, more and new viruses will be detected in the CNS tissue.

Although variations of the preparation protocols, sequencing methodology, and bioinformatic pipelines utilised for the studies included in this thesis have proven efficient for viral detection, including the detection of novel viruses, in a spectrum of organic materials by others in our laboratory (Cholleti *et al.* 2016; Karlsson *et al.* 2016; Malmberg *et al.* 2017; Cholleti *et al.* 2018a; Cholleti *et al.* 2018b; Öhlund *et al.* 2019; Hayer *et al.* 2021; Balinandi *et al.* 2022), it is reasonable to assume that there is room for improvements. With more time and resources, we could have explored other methods to identify more of the virome, *e.g.*, by DNA sequencing, increased sequencing depths, and different preparation protocols for the samples.

5.6.2 Metagenomic sequencing of semen pools (Paper IV)

After quality control and filtering, sequencing of the eight semen pools generated between 4.4 and 15.7 million reads per sample. About 24% of the reads mapped against the host genome (*Sus scrofa domesticus*) and were removed from the dataset. Of the remaining reads, approximately 98% could be taxonomically classified. Most of the classified reads, about 55%, were of microbial origin, mainly bacterial. Fewer than 1% of the reads were taxonomically classified as viral. Specific alignment against the APPV-genome identified APPV-sequences in one of the eight semen pools. At the contig level, approximately 94% of the *de novo* assembled contigs could be taxonomically classified. Most contigs, on average 60% in each pool, were of chordate origin, mainly from the *Sus scrofa domesticus*-genome. About 35% of the contigs were of microbial origin, primarily bacterial, and fewer than 1% of the classified contigs were of viral origin.

The output of the sequencing study represents the RNA microbiome, including the virome of extended semen intended for artificial insemination or the AI-dose's RNA microbiome that the females are exposed to while being inseminated. This RNA microbiome originates from the microorganisms of the semen, the reproductive tract, and the preputium, but also from microorganisms in the extender, including contaminants from the semen collection and the AI-dose preparation. Numerous post-ejaculation bacterial contaminants of boar semen have been described and discussed in

the literature (Althouse & Lu 2005; Althouse 2008; Althouse & Rossow 2011; Contreras *et al.* 2022). However, it was only recently discovered that the microbiome of AI-semen not only varies depending on the boars, but it varies between seasons (Zhang *et al.* 2020), boar studs, and semen preparation laboratories (Nitsche-Melkus *et al.* 2020). Interestingly, the hygienic conditions at the boar stud and the semen preparation laboratories may introduce up to 60 % of the contaminating bacteria that were detected in an AI-dose (Nitsche-Melkus *et al.* 2020).

Compared with the datasets produced from CNS tissue, the proportion of host-derived sequences was lower in the semen-derived datasets. This discrepancy may be attributed to the removal of sperm cells prior to RNA extraction. Still, most of the generated reads in the datasets were classified as *Sus scrofa domesticus*-genome. As expected, the semen-derived datasets contained a larger proportion of microorganisms, mainly bacteria, compared with the CNS-derived datasets. Although having a DNA-genome, the bacteria could be detected through RNA sequencing due to the ever-ongoing transcription in the cells.

Viral sequences in semen pools (paper IV)

Three viral species were detected in all datasets: Equine infectious anemia virus, Porcine type-C oncovirus, and Posavirus-1. Atypical Porcine Pestivirus generated 16 reads and two contigs in one dataset. Reads classified as different phages were also recovered from the datasets, but the overall viral occurrence was low.

The most common viral finding detected in all datasets was reads derived from the novel reagent-associated lenti-like virus misclassified as Equine infectious anemia virus (Porter *et al.* 2021). The second most common viral finding was reads classified as Porcine type-C oncovirus, also detected in all datasets. The only viral recovery of known clinical relevance was reads and contigs classified as APPV.

Reads classified as Posavirus were recovered from seven out of the eight datasets. Since Posavirus is associated with porcine stool and intestine, it cannot be determined if it is part of the virome of the reproductive tract, the semen, or if it represents faecal contamination (Chen *et al.* 2018a; Smoľak

et al. 2022). Compared with the viral spectrum of the CNS, the seminal RNA virome held a higher number of phage species, although at a low read count. The sparse viral occurrence and diversity in the semen support the assumption from human research of a naturally scant seminal virome (de Albuquerque *et al.* 2022). Further, data from a human study have indicated that a limited virus diversity in the semen is beneficial for achieving pregnancy (Gunderson *et al.* 2022).

Bacterial sequences in semen pools (paper IV)

Bacterial reads were recovered in the datasets from all semen pools. A majority of the reads and contigs were classified into three Gram-negative bacterial families. *Burkholderiaceae* (~ 20% of the bacterial reads, ~ 51% of the bacterial contigs), *Comamonadaceae* (~ 9% of the bacterial reads, ~ 6% of the bacterial contigs), and *Enterobacteriaceae* (~ 5% of the bacterial reads, ~ 1% of the bacterial contigs). About 18% of the bacterial reads and ~ 9% of the bacterial contigs were classified as so-called "uncultured bacterium"; *Ralstonia* sp. (*Ralstonia pickettii*, *Ralstonia insidiosa*, *Ralstonia solanacearum,* and *Ralstonia mannitolilytica*) being the most common bacterial genus. In this study, bacterial reads were detected in the negative RNA control *e.g.*, from the families *Burkholderiaceae* and *Comamonadaceae*, but at a significantly lower read count. Although no obligate pathogenic bacteria were detected, reads and contigs from bacteria with an opportunistic potential, such as *E. coli*, were recovered in all datasets.

No detection of antimicrobial resistance genes

The screening for antimicrobial resistance genes (ARGs) on the bacterial contigs did not identify the presence of any genes conferring resistance to antibiotics.

The dominance of Proteobacteria in the boar semen is in accordance with the results from the two other studies on the porcine seminal microbiome (Gòdia *et al.* 2020; Zhang *et al.* 2020), but while the studies from China and Spain detected both ARGs and pathogenic bacteria, no reads or contigs originating from obligate pathogenic bacteria or ARGs were recovered in the datasets derived from the Swedish boars. Instead, the microbiome obtained from Swedish boars is more similar to the human seminal microbiome than the

porcine seminal microbiome. The human seminal microbiome is diverse and consists of aerobic, facultatively anaerobic, and strictly anaerobic bacteria, including species considered to be opportunistic pathogens (Hou *et al.* 2013; Weng *et al.* 2014; Monteiro *et al.* 2018; Baud *et al.* 2019). Additionally, *Ralstonia* spp., which was abundant in the datasets obtained from Swedish boars, is the most prevalent species in studies on the human seminal microbiome (Hou *et al.* 2013; Weng *et al.* 2014; Baud *et al.* 2019).

Most bacteria detected in the boar semen, as well as most bacteria in human semen, such as bacteria from the families *Burkholderiaceae (e.g., Ralstonia* spp.*)* and *Comamonadaceae (e.g., Variovax* spp.*),* are ubiquitous bacteria commonly isolated in mammals but also the environment; in water, in soil, etc. However, bacterial sequences derived from water- and soil-associated genera, as well as organisms associated with humans, are well-known as contaminants of lab reagents and extraction kits, which might impact the common perception of the microbiome (Mohammadi *et al.* 2005; Salter *et al.* 2014a). These types of bacteria are also known as contaminants from semen collection and preparation (Althouse & Lu 2005; Althouse 2008; Althouse & Rossow 2011; Contreras *et al.* 2022). Nevertheless, it is reasonable to assume that these ubiquitous bacteria may colonise or contaminate the boars' reproductive tract and preputium and occur as contaminants in labs, kits, and reagents.

5.7 Atypical porcine pestivirus in wild boar (paper III)

This study included sera from 595 wild boars sampled in Sweden from the year 2000 to 2018. Of the sampled wild boars, 12 % (73/595) were APPV-genome positive in serum, and 72% (433/595) of the tested wild boars displayed APPV E^{rns}-specific antibodies. Most wild boars with APPV-genome in their blood also displayed a high antibody reactivity.

A general linear model, with serum reactivity (sample/positive control = S/P-value) as the response, and year, county, and season as the explanatory variable, was implemented. The model had an R^2 of 0.27 ($F(19, 394) = 7.385$, $p < 0.0001$). It could comprise 395 of the total 595 observations. The year and county were found to be significant predictors of serum reactivity

(S/P-value), but the season was not. The dataset on viraemic wild boars did not undergo any statistical tests due to the insufficient number of APPV-genome positive wild boars included. However, the data indicated that the proportion of viraemic wild boars fluctuated throughout the year. During autumn (September– November), the recorded genome prevalence was 13% (17/133) and 10% (13/125) during winter (December–February), compared with 5% (4/77) during spring (March–May), and 3% (2/69) during summer (June–August). A trend of a higher genome detection rate in the counties with the densest wild boar population, *i.e.*, Södermanland (29 %, 14/109) and Skåne (25%, 16/138), was recorded compared with the other counties (< 13%). Further, a significantly higher serum reactivity was seen among wild boars sampled in Södermanland compared with those sampled in other counties.

The pairwise comparisons by Tukey's honest significant difference test showed that wild boars from Södermanland, have a higher serum reactivity compared with wild boars originating from other counties. The serum reactivity in Södermanland ($n = 94$, S/P-mean value = 0.97) was significantly different ($p < 0.05$) from that in Halland ($n = 32$, S/P-mean = 0.58), Skåne ($n = 101$, S/P-mean = 0.7) and Blekinge ($n = 39$, S/P-mean = 0.77).

The dataset also indicated a difference in APPV-genome occurrence and serum reactivity towards APPV over the years. When comparing the years with > 80 observations (*i.e.*, the years 2005, 2009, 2013, 2017, and 2018), there seems to be a trend of an increasing proportion of APPV-genome positive animals ranging from 6% (6/100) in the year 2005 to 24% (20/82) in the year 2018. Pairwise comparisons made by Tukey's honest significant difference test showed that there was significantly higher serum reactivity in the year 2013 ($n = 104$, S/P-mean = 0.977; $p < .05$) than in 2002 ($n = 42$, S/P-mean = 0.489), and 2005 ($n = 95$, S/P-mean = 0.612). The genome detection rate appeared to have increased over time, and the counties with the densest population of wild boars, namely Södermanland and Skåne, had a higher genome detection rate and serum reactivity compared with the other counties. This result suggests that the spread of APPV is more intense in wild boar-dense areas.

The most important finding of this study was the high proportion of APPV-viraemic wild boars and wild boars with APPV-specific antibodies. So far, only a few studies are available for comparison on APPV in wild boars, but it may be noted that the detection rate of APPV-genome in wild boars varies across different countries. In studies from Sweden and Germany, the genome detection rate was high, 12 % and 19 %, respectively (Cagatay *et al.* 2018), compared to studies from South Korea, Spain, and Italy, where the genome detection rate was < 1% (Colom-Cadena *et al.* 2018; Sozzi *et al.* 2019; Choe *et al.* 2020). Further, a seroprevalence of 52% was recorded among German wild boars (Cagatay *et al.* 2018), but the seroprevalence was not investigated in the other studies. It is interesting to see this variance in APPV-genome detection rate and seroprevalence, as the wild boars included in all studies were free-ranging healthy animals sampled during the hunt.

The samples used in this study were obtained from hunting, which means that the selection process has an element of convenience sampling and not all years, counties, and seasons were proportionately represented. This sampling may affect the possibilities of data comparison between years, counties, and seasons. Still, the study indicated a variation in the genome detection rate and the proportion of seropositive wild boars across different years and counties in Sweden.

5.8 Phylogenetic analysis of APPV sequences (paper I and III)

For the phylogenetic analysis, a part of the NS3-encoding region of the APPV-genome was selected. For domestic pigs, the nucleotide identity of the sequences obtained from four farms ranged from 88.3% to 98.8%. The APPV-sequences from a piglet sampled in Dalarna 2012, (LR700964) and a piglet sampled in Blekinge 2017 (LR700966) shared the highest nucleotide identity. See Figure 3 for a map of the Swedish counties. The lowest nucleotide identity was seen between sequences obtained from the piglet sampled in Dalarna 2011 (LR700964) and a piglet sampled in Södermanland 2018 (LR700967).

The nucleotide identity between the five sequences obtained from wild boars ranged from 87.3% to 98.8%. The two sequences obtained from wild boars in Södermanland 2013, sampled in May and November, shared the highest nucleotide identity. The lowest nucleotide identity was seen between the sequences obtained from wild boars sampled in the year 2017 in Uppland and 2018 in Södermanland. The APPV-genome sequence originating from a wild boar sampled in the county of Södermanland in 2018, shared the highest nucleotide identity (96.4%) with an APPV-sequence obtained from an Italian wild boar sampled in the year 2020, and an APPV-sequence (95.8%) from a domestic pig sampled in the year 2018 in Södermanland. The other four wild boar APPV-sequences shared the highest nucleotide identity to an APPV-sequence obtained from a domestic pig sampled in Dalarna in the year 2011 (98.2-99.2%).

The genetic diversity of the APPV circulating in Sweden is visualised in the phylogenetic tree (Figure 9), in which the APPV sequences from Sweden fell into two distinct clusters. Most of the Swedish APPV sequences fell into Cluster I, forming one separate clade in the cluster, dominated by APPV sequences from Asia. Three Swedish APPV sequences fell into Cluster II, mostly dominated by APPV sequences from European pigs and wild boars.

A TempEst analysis was done, trying to estimate the time to the most recent common ancestor (TMRCA) of APPV in Sweden. No detailed estimation of the introduction date could be completed due to limitations in the temporal signal of the available data, but the TempEst analysis put the introduction of APPV into Sweden more than 100 years ago.

Figure 9. Phylogenetic tree from paper III comprising APPV sequences obtained from domestic pigs and wild boars

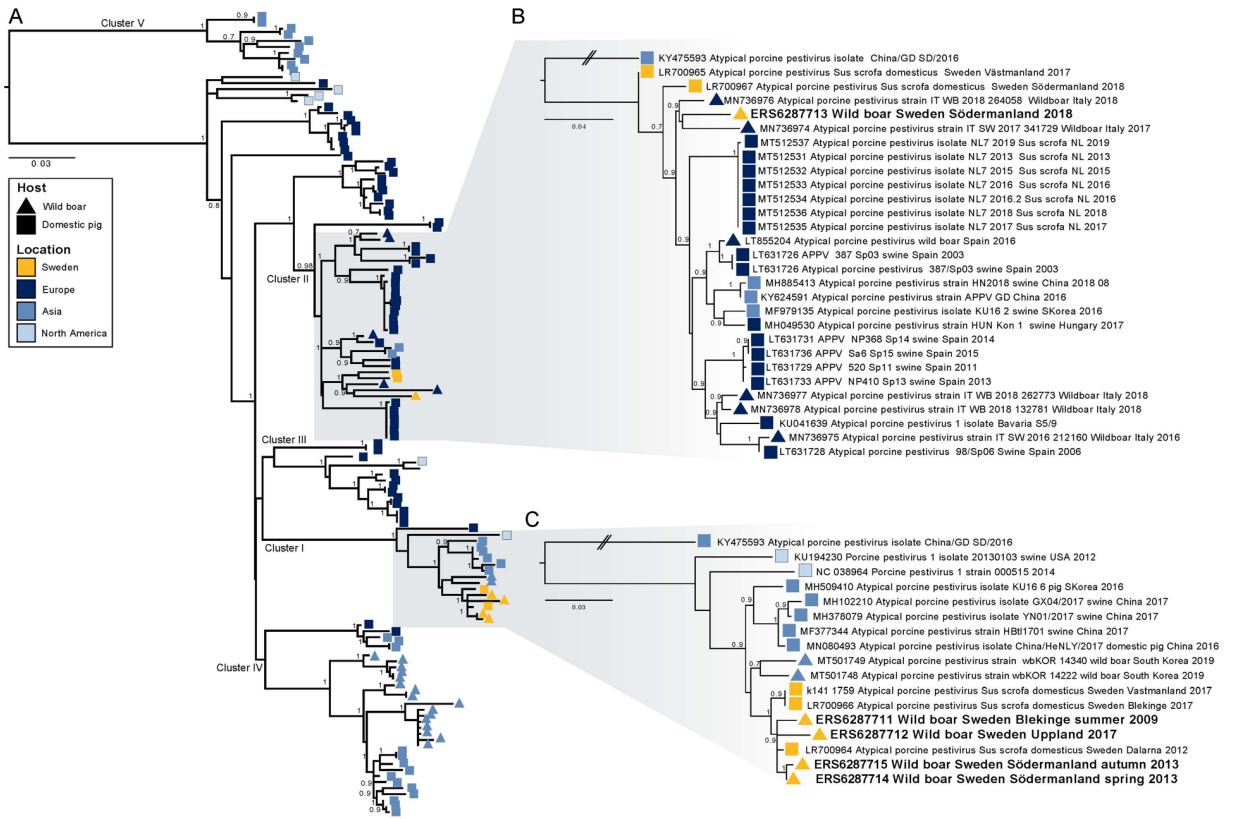
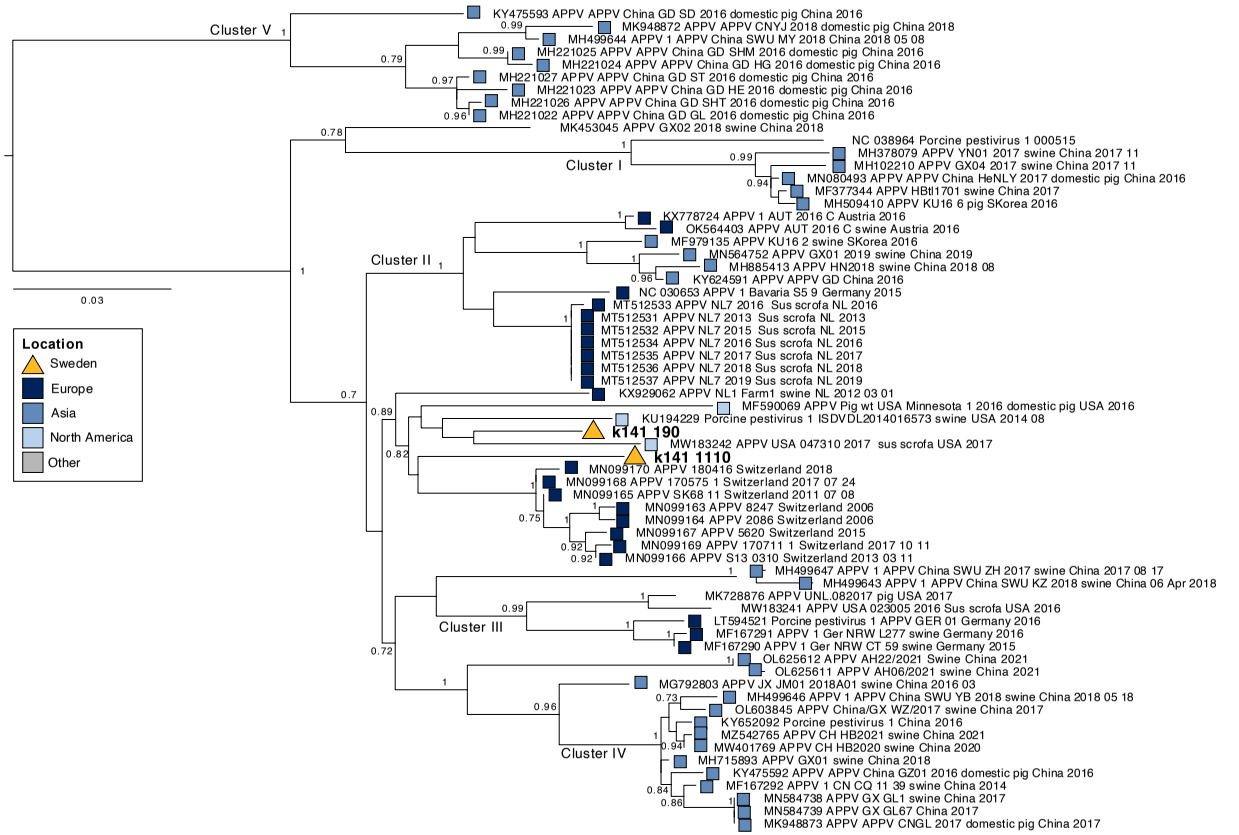


Figure 10. Phylogenetic tree from paper IV comprising APPV sequences obtained from domestic pigs and wild boars



The APPV sequences obtained from semen (paper IV) comprised the 5'UTR and spanned the glycoproteins E^{rns} and the first half of E1 of the APPV genome. One contig shared a 97. 6% sequence identity with an APPV-sequence retrieved from a domestic pig sampled in 2016 in the USA, whereas the other contig was most closely related to an APPV-sequence from a domestic pig collected in 2018 in Switzerland, with an identity of 95.2%. Phylogenetic analysis suggests that the APPV detected in semen is closely related to APPV sequences obtained from pigs in Europe and the USA (Figure 10). It would have been ideal to include sequences from the APPV genomes obtained from Swedish piglets and wild boars in the phylogenetic analysis, but these sequences could not be included as they spanned the NS3 region of the genome and the part of the APPV genome obtained from semen did not. However, previously sequenced Swedish viruses fell into Cluster I and Cluster II, indicating that the APPV obtained from piglets and wild boars is genetically distinct from the APPV recovered from the semen.

Reads classified as Posavirus-1 (paper IV) were detected in all semen datasets at a maximum of 194 reads in one dataset. Three contigs from dataset 4, ranging from 370-634bp and spanning the non-structural coding region (specifically the helicase), were further investigated using maximum likelihood phylogenetic analysis. Using the BLAST search tool, the obtained sequences were 90-95.8% similar to other Posavirus-1 sequences. In the phylogenetic tree, two contigs fell into a clade comprising sequences from pigs in Germany and the USA, and the third contig was sister to sequences from the USA and China but featured a long branch length. A limited number of Posavirus-1 sequences are available for comparison, none of which originates from Sweden. This lack of Posavirus sequences limits the phylogenetic analysis.

From the phylogenetic analysis of APPV, there appear to be at least three genetically distinct variants from the same genotype (Ren *et al.* 2022) currently circulating in Sweden. Two genetically distinct variants of APPV were found in domestic pigs and wild boar, and a third variant was detected in boar semen from a domestic pig. Concurrent circulation of two distinct variants, or the introduction of a new variant, was observed in the wild boar population of Södermanland: one variant was found in wild boar sampled in the year 2013 and another variant could be detected in domestic pigs and

wild boar sampled in 2018. The high genetic variation of APPV in Sweden, also within a small geographic area, aligns with the present literature (Sutton *et al.* 2022). This thesis only included sequencing data from a limited number of APPV genomes detected in Sweden, and more sampling and subsequent sequencing of APPV are urgent for a more profound knowledge of the molecular epidemiology of APPV.

It is crucial to have a better understanding of how APPV spreads in Sweden in order to establish effective biosecurity measures. Based on the current knowledge, there are two suggested routes of transmission of extra importance: 1) Into farms from breeding boars via AI-semen. APPV is readily transmitted via AI-semen from clinically healthy boars (Houston *et al.* 2022), and farmers are relying on pathogen-free semen to maintain good health status at their farms. 2) Between wild boar and domestic pigs (or vice versa). The detection of genetically close variants of APPV in domestic pigs and wild boars during the same time and/or geographic area *e.g.*, in Södermanland or Blekinge, indicates a transmission of the virus. Viral transmission between wild boars and domestic pigs in these areas is particularly concerning as Södermanland is one of the high-risk areas for the introduction of African swine fever (Chenais 2021). This emphasises the importance of thorough knowledge of how APPV is transmitted to take appropriate measures to prevent its spread.

6. General discussion including future perspectives on congenital tremor and APPV in Sweden

As a result of the studies included in this thesis, APPV could be established as the causative agent of CT type A-II in Swedish piglets. No relevant viral co-infections were identified in the CNS tissue of piglets with signs of CT type A-II and APPV was not detected in healthy piglets or piglets with signs of splay leg. Further, a high detection rate of APPV-genome and APPV-specific antibodies was observed in the Swedish wild boar population, and APPV-genome was discovered in commercial AI-semen.

In Sweden, there has been a generally low interest in congenital tremor in piglets among researchers and veterinarians. There are only a few reports on the subject, one from 1955 that discussed the epidemiology and suggested a viral cause (Larsson 1955), one from 1978 that described medical-induced CT following medical treatment with trichlorfon in pregnant sows (Bolske *et al.* 1978), one from 1986 indicating a low heritability for "trembling symptoms" (Lindström & Lundeheim 1986), and one from 2014 suggesting astrovirus as a possible cause to CT type A-II (Blomstrom *et al.* 2014).

Congenital tremor is not notifiable, despite its recurrent emergence at farms all over Sweden, and it is generally not included in farm or veterinary records. Consequently, the prevalence and case fatality rate attributed to the disease in Sweden is unknown. However, given the high piglet mortality in Sweden (Gård&Djurhälsan 2022), CT type A-II should not be overlooked without a thorough analysis of its prevalence and impact on piglet health and survival.

Knowledge of the causative agent is a cornerstone for surveillance and control. Therefore, now that APPV has been identified as the causative agent of CT type A-II (Hause *et al.* 2015; Arruda *et al.* 2016; de Groof *et al.* 2016; Postel *et al.* 2016) and has been detected in Sweden (Stenberg *et al.* 2020b), CT type A-II and its' causative agent are easier to address. Since APPV has an immunoregulatory effect on the host (Mou *et al.* 2021; de Martin & Schweizer 2022; Stenberg *et al.* 2022), can induce persistent infections (Schwarz *et al.* 2017), and may circulate on a farm for ages once introduced (Folgueiras-González *et al.* 2020a), control of the virus should be urgent for the porcine industry.

The findings presented in this thesis indicate that there are at least two ways in which APPV may be transmitted to pigs in Swedish farms: through AI-semen (discussed in Paper IV) and through wild boars (discussed in Paper III). The importance of these suggested transmission routes remains, however, to be investigated, and further studies are needed. As wild boars approaching farms and the reliance on commercial boar semen for breeding are risk factors that apply to most farms, it is important to promptly address these two potential transmission routes. It is also possible that the trade of live animals could contribute to the spread of APPV, but further research is needed to understand the epidemiology of this virus in Sweden.

In Sweden, artificial insemination is the primary breeding method at commercial pig farms. Given that APPV may be transmitted via semen and artificial insemination (Houston *et al.* 2022), all farms are at risk of introducing APPV as long as the virus is not controlled for at the boar stud. Today, there is no commercial testing for APPV in place, but following our detection of APPV in semen intended for artificial insemination, efforts are being made to make APPV-testing available in Sweden.

Pig farmers in Sweden are facing the challenge of wild boars rapidly increasing in numbers and spreading across the country. In some wild boar-dense areas, the transmission of pathogens between wild boars and domestic pigs, *e.g.*, APPV (Stenberg *et al.* 2021) and *Salmonella* Choleraesuis (Ernholm *et al.* 2022), has already been indicated. Therefore, identifying and addressing possible transmission routes from wild boars to domestic pigs is crucial. Transmission via *e.g.*, direct contact, indirect contact, course fodder,

bedding material, or water, should be investigated and tended to accordingly. Knowledge of the transmission routes may also help improve biosecurity measures at pig farms, given that APPV could serve as a proxy for other pathogens, such as the African swine fever virus (ASFV). If APPV can be transmitted from wild boars to domestic pigs, so can ASFV. By identifying and preventing the transmission routes of APPV between wild boars and domestic pigs, the overall biosecurity at pig farms will be significantly improved.

Controlling the spread of APPV will be challenging due to its complex nature, which involves multiple transmission routes, persistent infections, intermittent shedding, subclinical disease, and wild boar as potential vectors. To control the spread of APPV, a test for viral detection should be the priority, and APPV should be part of the pathogens testing panel used at the boar stud quarantine. By ensuring APPV-free AI-semen, one apparent transmission route would be interrupted. For solid testing results and future control of the virus, additional knowledge about the pig's immunity towards APPV, shedding, and persistent APPV infections is needed. A comprehensive understanding of the epidemiology of APPV on farms and among wild boars is also necessary for effective control of the viral spread. With all these pieces in place and if stakeholders are involved, controlling APPV among domestic pigs should be possible.

7. Concluding remarks

In this thesis, Atypical porcine pestivirus was identified in piglets with signs of congenital tremor, Swedish wild boars, and semen intended for artificial insemination. In addition, the tissue-resident CNS virome and boar semen microbiome, including the virome, were studied. Based on the results included in this thesis, the following specific conclusions can be drawn:

- APPV is present in the brain tissue of Swedish piglets with signs of congenital tremor.

- Ultrastructural lesions such as degenerated mitochondria of the oligodendrocytes in the cerebellum seem to be associated with APPV infection.

- A sparse tissue resident virome of the brain and the spinal cord of piglets is indicated.

- A STING-associated cytokine profile appears to be induced in the porcine brain tissue during infection with APPV.

- APPV has been present in domestic pigs in Sweden since at least 2004 and in the wild boar population since at least 2000.

- APPV circulating within Sweden is genetically diverse, but in some cases, a genetic similarity between APPV found in wild boars and domestic pigs is seen.

- APPV genome can be excreted in urine and saliva from piglets with signs of congenital tremor type A-II.

- APPV can be detected in AI-doses from boars.

- No infectious agent could be associated with splay leg in Swedish piglets using high throughput sequencing.

In summary, the knowledge generated from the studies included in this thesis may facilitate the development of a future control program for APPV among domestic pigs in Sweden.

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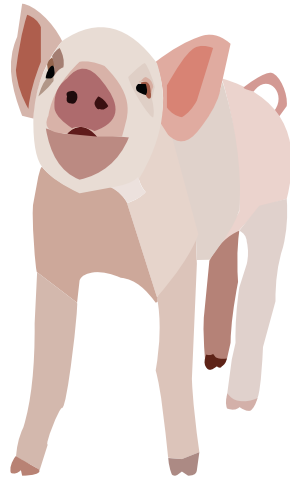

Popular science summary

Congenital tremor and splay leg are diseases affecting the nervous system of newborn piglets. Both conditions are relatively common and seen in piglets globally. Currently, there are no laboratory tests to diagnose either congenital tremor or splay leg. Instead, the diseases are diagnosed based on the clinical presentation. Piglets with congenital tremor display a varying degree of shaking of the head and body, sometimes combined with uncoordinated leg movements. Piglets with splay leg, cannot pull their back legs together in order to stand up. Often, it is only the hind legs that are affected by splay leg. If both the front and hind legs are splayed, the piglet will be immobile, lying flat on its stomach. Neither congenital tremor nor splay leg is fatal *per se*, but as piglets with these syndromes have problems moving, there is a high risk of them being hurt or crushed by the sow. These piglets are also at risk of malnourishment and starvation as their impaired mobility can make it difficult to get to the udder to nurse.

In Sweden, both congenital tremor and splay leg occur in pig herds all over the country. Currently, several different viruses, toxins, medications, and gene defects are known that, when pregnant sows are exposed, can induce splay leg and various types of congenital tremor in piglets. In Swedish pigs, a variant of congenital tremor called congenital tremor type A-II is occurring. The causative agent of congenital tremor type A-II was unknown for decades. Then, in 2015, Atypical porcine pestivirus (APPV) was detected and in 2016 associated with congenital tremor type A-II. There were also indications of an association between APPV and splay leg.

The thesis aimed to determine whether APPV causes congenital tremor type A-II or splay leg in Swedish piglets and to investigate other viral causes of tremor type A-II or splay leg in pigs in Sweden.

Using molecular methods (qPCR), APPV could be detected in brain tissue from piglets with symptoms of tremors type A-II. In piglets with congenital tremor or healthy control pigs, APPV was not present. In addition, deep sequencing of the material from the piglets was performed to investigate the presence of other viruses. However, no viruses other than APPV were discovered. Further, to begin to understand the epidemiology of APPV in Sweden, studies on APPV in wild boar and semen were also performed. Based on the results from these studies, it is likely that wild boar and AI-semen may contribute to the spread of APPV.

In summary, the studies show that APPV commonly occurs in pigs and wild boars in Sweden. However, no evidence of virus-induced splay could be found. The results presented in the thesis may be used as a basis for a control program to limit the spread of APPV in Sweden, e.g., by including APPV in the quarantine pathogen screening of future breeding boars.

Populärvetenskaplig sammanfattning

Skaksjuka och fläksjuka är sjukdomar som drabbar nervsystemet hos nyfödda griskultingar. Båda sjukdomarna är relativt vanligt förekommande och ses hos grisar över hela världen. I dagsläget finns inga laboratorietester för att diagnostisera vare sig skak- eller fläksjuka, utan sjukdomarna diagnostiseras utifrån den typiska sjukdomsbilden. Hos griskultingar med skaksjuka ses skakningar av varierande allvarlighetsgrad, ibland kombinerat med okoordinerade benrörelser. Griskultingar med fläksjuka har istället problem med att dra ihop benen för att kunna resa sig upp. Oftast är det bakbenen som drabbas av fläkning men ibland ses samtidig fläkning av både fram och bakben. Om både fram- och bakbenen är drabbade blir griskultingen liggande orörlig, platt på magen. Vare sig skak- eller fläksjuka är i sig dödliga men då kultingar med dessa problem har svårt att röra sig, ökar risken för att de blir klämda/ihjällegade av suggan. De riskerar också att drabbas av näringsbrist eller att dö av svält då rörelseproblemen kan göra att de har svårt att ta sig fram till juvret för att dia.

I Sverige förekommer både skak- och fläksjuka i grisbesättningar över hela landet. I dagsläget är flera olika virus, gifter, mediciner och gendefekter kända som, när dräktiga suggor exponeras, kan orsaka olika typer av skak- eller fläksjuka hos kultingar. Hos svenska grisar ses varianten skaksjuka typ A-II, en variant som länge misstänkts vara orsakad av virus. År 2016 kunde det i USA nyupptäckta viruset, Atypisk porcint pestivirus (APPV), sättas i samband med skaksjuka typ A-II. Då sågs även tecken på att APPV skulle kunna framkalla fläksjuka.

Målet med avhandlingen var att undersöka om APPV orsakar skak- eller fläksjuka hos svenska griskultingar samt att kartlägga andra eventuella virala orsaker till skaksjuka typ A-II och fläksjuka i Sverige.

Med hjälp av molekylära metoder (qPCR) kunde APPV påvisas i hjärnvävnad från griskultingar med symptom på skaksjuka typ A-II. Hos griskultingar med fläksjuka eller hos friska kontrollgrisar hittades inte APPV. Djupsekvensering av materialet från kultingarna gjordes också för att undersöka om det fanns andra virus hos griskultingarna men inga virus förutom APPV hos de skakande grisarna hittades. Studier på APPV hos vildsvin samt i sperma hos galtar gjordes även för att börja kartlägga hur spridningen av APPV sker i Sverige. Utifrån dessa studier är det troligt att både vildsvin och sperma bidrar till spridningen av APPV.

Sammanfattningsvis visar studierna att APPV förekommer frekvent hos grisar och vildsvin i Sverige samt att viruset orsakar skaksjuka typ A-II. Inga tecken på virusorsakad fläksjuka sågs dock. Resultaten av studierna i avhandlingen kan ligga till grund för kontrollprogram för att begränsa spridningen av APPV i Sverige, till exempel genom att inkludera APPV i karantänprovtagningen blivande semingaltar.

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I

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RESEARCH ARTICLE Open Access

Detection of atypical porcine pestivirus in Swedish piglets with congenital tremor type A-II

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Abstract

Background: Congenital tremor (CT) type A-II is a neurological disorder characterized by tremor of the head and body of newborn piglets. The suggested causative agent of the disease is the recently found atypical porcine pestivirus (APPV). The virus has been detected in piglets suffering from congenital tremor in central Europe, South and North America and in China but no studies has so far been performed in the Nordic countries. The overarching goal of this study was to investigate if APPV is present in the brain tissue of Swedish piglets suffering from congenital tremor.

From June 2017 – June 2018, 15 piglets from four Swedish farms with ongoing outbreaks of congenital tremor and 13 piglets with splay leg originating from four different farms, were investigated for presence of APPV RNA in brain tissue. Matched healthy control piglets ($n = 8$) were also investigated. Two APPV-specific RT-qPCR methods targeting the NS3 and NS5B region, respectively, were used. A retrospective study was performed on material from Swedish piglets with congenital tremor sampled in 2004 ($n = 11$) and 2011/2012 ($n = 3$) using the described APPV-specific RT-qPCR methods. The total number of piglets with signs of CT in this study was 29.

Results: Atypical porcine pestivirus-RNA was detected in 93% (27/29) of the piglets suffering from congenital tremor. All piglets with congenital tremor from 2004 ($n = 11$) and 2012 ($n = 3$) were PCR-positive with respect to APPV, whereas, all of the healthy controls (n = 11) were negative. The piglets with congenital tremor sampled 2017–2018 had an odds ratio of 91.8 (95% CI 3.9128 to 2153.7842, z = 2.807, $P = 0.0050$) to test positive for APPV by qRT-PCR compared to the healthy piglets (Fishers exact test $p < 0.0001$). These findings make it interesting to continue investigating APPV in the Swedish pig-population.

Conclusion: This is the first description of atypical porcine pestivirus in piglets suffering from congenital tremor type A-II in Sweden and the Nordic countries. The virus has been present in the Swedish pig population since at least 2004.

Keywords: Congenital tremor, Type A-II, Atypical porcine pestivirus, Splay legs, Sweden, Swine, Piglets

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Background

Congenital tremor (CT) is a neurological disorder that affects newborn piglets. The disease is characterized by tremors of the head and body, and may in severe cases be complicated by ataxia that exacerbates the piglets' ability to move and suckle, resulting in reduced growth rate or increased pre-weaning mortality [1–3]. The neurological signs are caused by impaired saltatory transmission due to hypomyelination of the central nervous system (CNS) [3–5]. At present, congenital tremor is divided into six sub-types: type AI-AV and a B-type. By definition, all A-forms have hypomyelination of the CNS as the main histopathological finding, whereas the B-form presents with identical clinical signs but without histopathological lesions [1, 3–5].

For over half a century, congenital tremor type A-II was attributed to an unidentified virus [2], but in 2016 it was associated with the recently discovered atypical porcine pestivirus (APPV) [2, 6–8]. Atypical porcine pestivirus is a ssRNA+ virus that belongs to the species *Pestivirus K* in the genus *Pestivirus* within the family *Flaviviridae* [9, 10]. The genome of APPV is ~ 10.8–11.5 kb and up until now about 20 complete genomes have been published [11]. Interestingly, the genetic variability of the APPV isolates is very high also within the same geographic region [6, 12].

In two independent experiments with pregnant sows, APPV PCR-positive material was used to induce congenital tremor in piglets [7, 8]. Since the discovery of APPV in the US [9], the virus has been described in both diseased and healthy domestic pigs in Europe, Asia and in South and North America [6, 9, 13–18] and in serum as well as faecal samples from wild boars sampled in central Europe [14, 19, 20]. Atypical porcine pestivirus has also been detected in stored material from historical outbreaks of congenital tremor the oldest detection currently being from samples stored in 1997, originating from Spanish piglets suffering from congenital tremor [14].

Interestingly, in the experimental infections described above, splay leg was induced in 0–40% of the piglets born from the sows inoculated with APPV [7, 8]. This prevalence was an unusual observation of splay leg since the syndrome typically occurs only as sporadic cases [21], whereas congenital tremor commonly causes distinct, high-morbidity outbreaks [2, 15, 22].

Splay leg is characterized by impairment of the adducting muscles of the hindlimbs and, in severe cases, of the forelegs as well. This is attributed to hypomyelination of the spinal cord and the nerves innervating the affected muscles [23, 24]. The syndrome was first described in 1967 [23] and the hitherto proposed causal factors are numerous e.g., heritable gene defects, viral infections, various management factors, nutrition, and, fusarium toxin in the feed of the pregnant dam [21, 25–27].

There are three primary aims of this study: (1) to investigate the presence of APPV in brain tissue from Swedish piglets affected by congenital tremor or splay legs sampled 2017–2018; (2) to perform a retrospective study of the presence of APPV in historical material from Swedish piglets affected by congenital tremor; and (3) to perform a phylogenetic analysis of the obtained APPV sequences.

Results

Detection of APPV in piglets with congenital tremor

Atypical porcine pestivirus-RNA was detected by qRT-PCR in 93% (27/29) of the piglets suffering from congenital tremor. All piglets exhibiting signs of congenital tremor from 2004 ($n = 11$) and 2012 ($n = 3$) were PCR-positive with respect to APPV. Of the piglets sampled in 2017–2018, 87% (13/15) of the samples were positive with respect to APPV. Interestingly, the two piglets with congenital tremor that were negative with respect to APPV originated from the same farm (Farm F) sampled in 2018. All of the healthy controls sampled in 2017–2018 ($n = 8$) and the healthy controls sampled in 2012 ($n = 3$) were PCR-negative with respect to APPV.

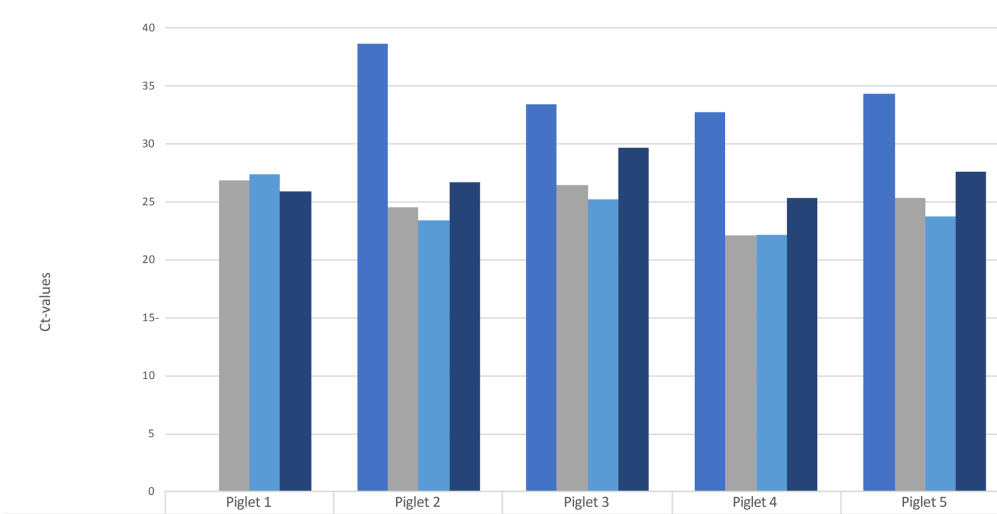
The piglets with congenital tremor sampled 2017–2018 had an odds ratio of 91.8 (95% CI 3.9128 to 2153.7842, z = 2.807, $P = 0.0050$. MEDCALC®) to test positive in brain tissue for APPV by qRT-PCR as compared to the healthy piglets. Fishers exact test for the same sample gave a $p < 0.0001$ (Sergeant, ESG, 2019. Epitools epidemiological calculators. Ausvet Pty Ltd. Available at http://epitools.ausvet.com.au).

To get a clear overview of the Ct-values, all values were ranked in accordance with other publications; Ct-values (cycle quantification values) were graded as high (Ct < 28), moderate (Ct 28–33) and low (Ct 33–40) [14, 28]. Table 1 presents the mean Ct-values obtained from the piglets at each farm. The specific Ct-values obtained from each piglet are presented in the supplementary material in the file "cq_values_congenital_tremor_appv.docx".

As shown in Fig. 1, APPV-RNA was also detected by qRT-PCR in urine (in 4/5 piglets) and in saliva (in 5/5 piglets) from piglets with signs of congenital tremor.

Table 1 A summary of the Ct values obtained from the piglets with congenital tremor from the six farms

Farm	Year	Location	Number of pigs	Ct-value
A	**2004**	Central Sweden	n = 11	low
B	**2012**	Central Sweden	n = 3	low – high
C	**2017**	Central Sweden	n = 3	high
D	**2017**	South of Sweden	n = 5	moderate – high
E	**2018**	Central Sweden	n = 5	high
F	**2018**	Central Sweden	n = 2	undetectable



	Piglet 1	Piglet 2	Piglet 3	Piglet 4	Piglet 5
NSB3 Mean Ct urine	0	38,69	33,41	32,72	34,29
NSB3 Mean Ct saliva	30,2	27,62	29,79	24,87	28,48
NSB3 Mean Ct brain	27,43	23,37	25,22	22,19	23,74
NS5B Mean Ct brain	25,89	26,72	29,64	25,38	27,59

Fig. 1 Five piglets with congenital tremor collected at farm E were screened for APPV-RNA in urine and saliva. RNA from atypical porcine pestivirus was detected with qRT-PCR in the saliva from 5/5 piglets and in the urine from 4/5 piglets. The Ct-values obtained from the brain-tissue are presented for comparison

Sequence and phylogenetic analysis

The four samples from Farm B, C, D and E generated between 775 and 812 nucleotides from the APPV NS3 protein: Farm B (ID: LR700964, accession ERS3734195) 775 bp, Farm C (ID: LR700965, accession ERS3734196) 807 bp, Farm D (ID: LR700966, accession ERS3734197) 810 bp and Farm E (ID: LR700967, accession ERS3734198) 812 bp. All generated sequences have been deposited at the European Nucleotide Archive at EBI. The nucleotide identity among the four sequences ranged from 88.3 to 98.8%. The sequence Farm C (ID: LR700965) and Farm B (ID: LR700964) shared the highest identity at the nucleotide level at 98.8%.

Both Farm C (ID: LR700965) and Farm B (ID: LR700964) displayed the highest identity at the nucleotide level, 97.3%, to two sequences obtained from Chinese domestic pigs (GenBank accession MH378079.1 and MH509410.1). The other two Swedish sequences, Farm D (ID: LR700966) and Farm E (ID: LR700967), shared 97.4% identity at the nucleotide level. These two sequences, from Farm D and Farm E, both shared the highest nucleotide sequences identity, 96.2 and 95.6%, respectively, with a sequence obtained from a Spanish wild boar (GenBank accession LT855204.1).

In the phylogenetic tree the Swedish sequences (marked * in Fig. 2) clustered with sequences from domestic pigs from China as well as with wild boar from Germany.

Pathology

Of the 15 piglets sampled 2017–2018, all piglets with congenital tremor and the healthy controls had milk in their ventricles, whereas the ventricles of the piglets with splay leg were empty. No gross lesions were recorded at necropsy. Necropsies were also performed on all 11 piglets from 2011 but no gross lesions were recorded. These 11 piglets were also PCR-negative for PCV-2.

No necropsies were done on the 6 piglets sampled 2012, although the brains were subjected to histopathological investigation. In the piglets with clinical signs of CT, mild to moderate vacuolar changes of the white matter were observed in the cerebrum, brain stem, and cerebellum [29].

No detection of APPV in piglets with splay leg

All of the piglets with splay leg ($n = 13$) sampled in 2017–2018 were PCR-negative in the brain tissue with respect to APPV.

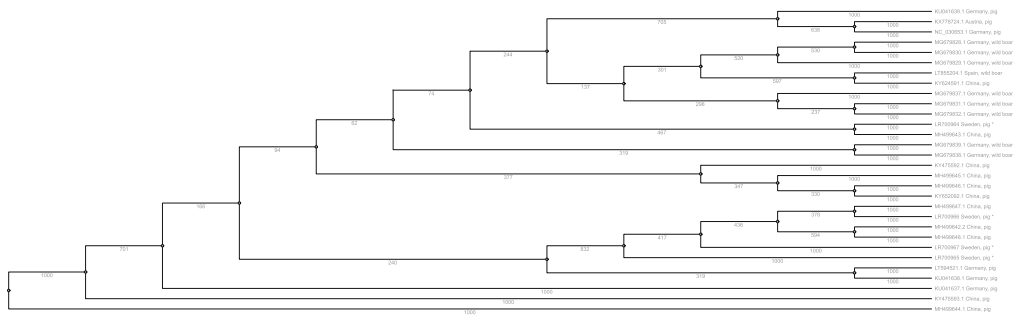


Fig. 2 Phylogenetic tree of APPV-sequences, the Swedish sequences marked * cluster with sequences from domestic pigs from China as well as with wild boar from Germany

Discussion

This is the first description of APPV in piglets with congenital tremor type A-II in Sweden and in the Nordic countries. Atypical porcine pestivirus was detected by qRT-PCR in 27/29 samples of brain tissue obtained from 29 piglets with congenital tremor. The clinical samples were collected from five different farms between 2004 and 2018. Hence, this study provides evidence of APPV being present in Swedish pigs with congenital tremor type A-II since at least 2004. The virus was, however, not detected in the brain tissue of the healthy control piglets or the piglets with splay leg. Thus, in this study we found no evidence for APPV as the causative agent of splay leg in Swedish piglets.

One previous report has suggested astrovirus as a possible causative agent of congenital tremor type A-II in Swedish piglets but this report should be interpreted with caution since the virus was detected in both healthy and diseased animals [29]. However, the presence of APPV in the brain tissue of these astrovirus-positive piglets with congenital tremor and absence of APPV in healthy piglets provides evidence of astrovirus being an incidental finding, or being present as a co-infection, rather than the causative agent.

There are some proposed differential diagnoses to tremor in piglets e.g. PMWS, Aujeszky's disease, porcine reproductive and respiratory syndrome (PRRS), aflatoxicosis, classical and African swine fever. Since Sweden has active surveillance programs and are deemed free from for Aujeszky's disease, porcine reproductive and respiratory syndrome (PRRS) as well as classical and African swine fever, and PMWS (PCV-2) not are supposed to cause congenital tremor [30–33] we speculate that APPV is a causative agent of congenital tremor in Swedish piglets as well as in other countries [34–36].

The shedding of atypical porcine pestivirus in urine and saliva is in line with other publications [8, 15], but our study is the first report where a commercial viral swab was used. When more details of the shedding of APPV are identified, saliva sampling could in the future be an easy and cost-effective way to screen large groups of pigs for the virus.

The analysis of the sequences obtained from the APPV-positive piglets confirms the findings by others, that APPV is a genetically variable virus with no clear geographic clustering [37]. Interestingly, the Swedish sequences show nucleotide identity not only to sequences from domestic pigs in China but also to sequences from APPV in wild boars in Spain and Germany. Hence, a screening and phylogenetic analysis of APPV in the Swedish wild boar population would be highly interesting when trying to elucidate a possible route of transmission within Europe.

Since this is the first description of APPV in piglets with congenital tremor type A-II in Sweden, further studies are needed to determine the prevalence of APPV in the Swedish pig population. In addition, the occurrence as well as the mechanism of potential co-infections of other viruses and APPV should be investigated.

Conclusion

This is the first description of atypical porcine pestivirus in piglets with congenital tremor type A-II in Sweden and the Nordic countries. The virus has been present in the Swedish pig population and been causing congenital tremor in piglets since at least 2004. Interestingly, the virus was not detected in piglets suffering from splay leg or in the healthy control piglets.

Methods

Clinical cases and sample collection

All animal studies were approved by the ethical committee of Uppsala 2017-02-10 (Dnr 5.8.10–00431/2017) and the owners of the herds gave informed consent prior to the start of the study.

During the period from June 2017 to June 2018, 15 piglets were obtained from four Swedish farms with ongoing outbreaks of congenital tremor. Of these piglets, 13 piglets were aged 1–2 days and two piglets were aged 5 days. All piglets were in good general condition with moderate to severe signs of congenital tremor. Three of the four farms were located in the central part of Sweden, with the remaining farm being located in the south of Sweden. The farms are marked on the map in Fig. 3. During the same period, 13 piglets aged 1–2 days old suffering from splay leg were obtained from four different farms located in the central part of Sweden. Most of these piglets had decreased demeanour. Piglets from the same farms and sows at their next farrowing were included as healthy controls; eight 1-day-old piglets in good condition were obtained. In cases where the original sow was unavailable, a piglet born to a sow from the same farrowing group was sampled. None of the sampled farms had any documented contact with each other and the outbreaks were separated in time, or had simultaneous outbreaks of congenital tremor and splay leg.

The piglets were transported to the pathology section at the Swedish University of Agricultural Sciences in Uppsala, Sweden. The piglets were sedated with an intramuscular injection of tiletamine and zolazepam (Zoletil®, Virbac, Carros, France) and a blood sample was obtained from the jugular vein. From the five piglets originating from farm E urine and saliva were also collected during sedation using commercial ESwabs (Copan Italia Via Perotti, Italy). All piglets were euthanized by an intraperitoneal injection of pentobarbital (Allfatal vet. Apotek Produktion & Laboratorier AB, Malmö, Sweden) with necropsy being performed within minutes.

Samples from the brain, spinal cord, saliva, urine, hearth, lung, quadriceps muscle, kidney, liver, spleen, ventricle, duodenum, jejunum, ileum, caecum, and colon were sampled and immediately put on dry ice. The tissue samples were then stored at − 80 °C. Corresponding tissue samples were fixed in 10% formaldehyde for future studies.

Retrospective study

A retrospective study was carried out on material from piglets sampled in 2004 ($n = 11$) and 2011/2012 ($n = 6$). The samples from 2004 consisted of serum originating from eleven piglets affected by congenital tremor. The samples were collected from one litter of piglets originating from a farm located in the central part of Sweden. Necropsies were performed on all 11 piglets from 2004 with no records of gross lesions. These 11 piglets all tested negative for PCV-2.

The samples from 2012 consisted of brain tissue collected at the end of 2011 and beginning of 2012 from piglets on one farm during an ongoing outbreak of congenital tremor. Three newborn piglets with congenital tremor were euthanised and sampled at the farm. When the outbreak had ceased, three healthy newborn control animals from the same farm were euthanised similarly. The brains were collected and subjected to histopathological investigation, but no complete necropsies of the

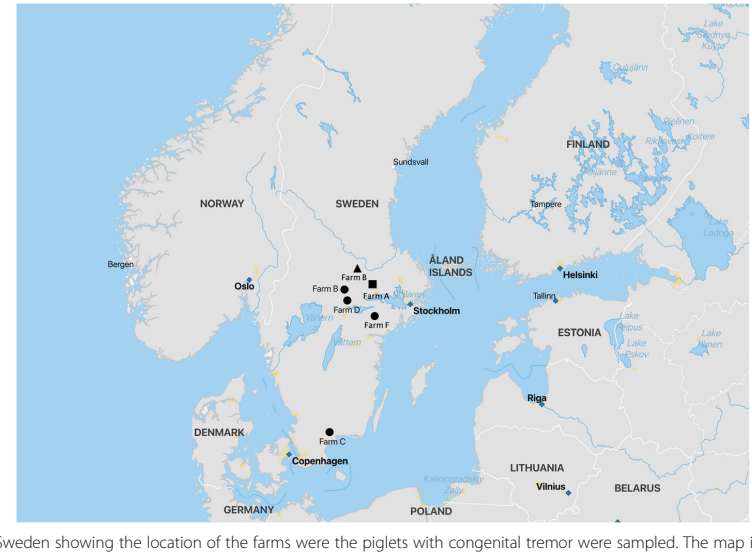


Fig. 3 A map of Sweden showing the location of the farms were the piglets with congenital tremor were sampled. The map is made using QGIS (QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.org)

bodies were performed. Brain tissue from all six piglets tested PCR-positive with respect to porcine astrovirus [29]. In the piglets with clinical signs of congenital tremor, mild to moderate vacuolar changes of the white matter were observed in the cerebrum, brain stem, and cerebellum [29].

Both the serum and brain samples were stored at −80 °C for future investigations.

Sample preparation, RNA isolation, qRT-PCR (quantitative reverse transcription-PCR) and sequence analysis

The brain samples were cryolyzed using a Precellys tissue homogenizer (Bertin Corp. Rockville, MD, USA), RNA was extracted from all samples through a trizol-phenol-chloroform protocol, and cleaned using the GeneJET RNA kit (ThermoFisher Scientific, Waltham, MA, USA). In addition, RNA from the sera was extracted using the same protocol but without homogenization. The APPV genome was detected using an APPV-specific RT-qPCR protocol based on the QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany) as described by [6] with a primer-pair targeting the NS3 encoding region of the APPV genome. The assay was run in duplicate under standard conditions on a Bio-Rad CFX96™ Real-time system in a C1000 Touch™ thermal cycler (Bio-Rad, Hercules, CA, USA) with a plasmid containing the NS3 encoding region of the APPV genome as a positive control. One primer and one probe, denoted "Swe" in Table 2, were slightly modified as compared to the protocol by [6] in accordance with [13], to better match the only described sequence of Porcine pestivirus in Sweden. All the samples were also analysed by an APPV-specific RT-qPCR targeting the non-structural protein NS5B in accordance with [12]. The RT-qPCR was run in duplicate under standard conditions using the qScript XLT One-Step RT-qPCR ToughMix (Quanta Biosciences, Gaithersburg, USA) on the above-mentioned Bio-Rad CFX96™ Real-time system in a C1000 Touch™ thermal cycler (Bio-Rad, Hercules, CA, USA).

Table 2 Primer and probe sequences used for qRT-PCR APPV detection, in accordance with previously published protocols, primers and probes [6, 12]. The primer and probe, denoted "Swe" are slightly modified in accordance with [13]

Oligo name	Sequence (5′-3′)
APPV-NS5B-303F	GTAGGGCGGATACAGAAATA
APPV-NS5B-385R	GGYACTTCCTCCATCATGG
APPV_5587-fw (NS3)	CAGAGRAAAGGKCGAGTGGG
APPV_5703_Swe-rev (NS3)	ACCATACTCTTGRGCCTGCAG
APPV_5087-fw (NS3)	GAAAGTGTCTGCCGCTTCATG
APPV-NS5B-336-FAM	AAATATTGGAAATYYATTGACAATTTGAC
APPV_Swe probe	ACTACTATCCTTCGGGGGTRGTRCCGA

Sequence analysis

From each APPV-positive farm, the sample with the lowest Ct-value was selected for sequencing. A part of the NS3-gene was PCR-amplified using the primers APPV_5087-fw and APPV_5703_Swe-rev with the Invitrogen™ SuperScript™ IV One-Step RT-PCR-System, using the ezDNase™ Enzyme protocol (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. The product was run on a 2% agarose gel stained with GelRed, visualized by UV-transillumination (GelDoc, Bio-Rad Laboratories, Inc., Richmond CA, US), purified using the Thermo Fisher Scientific GeneJET Gel Extraction Kit) and Sanger-sequenced at Macrogen Inc. Europe (Amsterdam, NL).

To get a clear and readily understood format of the tree, the phylogenetic analysis was performed on 26 full and partial genome sequences covering the APPV NS3 sequences extracted from the GenBank. The tree was constructed using the MAFFT alignment tool and the PHYLIP Neighbor-Joining method with a bootstrap value of 1000 using the UGENE software [38]. A bayesian tree were also made using the MR Bayes tool within the UGENE software [38]. To confirm the tree's constitution and clustering, additional Neighbor-Joining trees were constructed, as well as Bayesian trees made with the MR Bayes tool within the UGENE software [38]. The bayesian trees and the Neighbor-Joining trees were consistent with each other.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12917-020-02445-w.

Additional file 1.

Abbreviation

APPV: Atypical porcine pestivirus

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Authors' contributions

All authors (HS, MJ, MM) designed the experiments; MM designed the primers for Real-Time qPCR; HS performed the experiments; all authors (HS, MJ, MM) analyzed the data; HS wrote the original draft. All authors (HS, MJ, MM) read and approved the final manuscript.

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Availability of data and materials

The specific QC-values are published in the supplementary material. The sequences analyzed during the current study are available at the European Nucleotide Archive at EBI under accession numbers LR700964- LR700967.

Ethics approval and consent to participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. All animal studies were approved by the ethical committee of Uppsala 2017-02-10 (Dnr 5.8.10–00431/2017) and the owners of the herds gave informed consent prior to the start of the study. The pigs included in the research were purchased from the farms and thus the farmers' provided a verbal informed consent to sell the pigs for the research purpose stated. This procedure was approved by the Uppsala Ethical committee before the start of the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interest involved in the completion of this study.

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Publisher's Note

II

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RESEARCH

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Congenital tremor and splay leg in piglets – insights into the virome, local cytokine response, and histology

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Abstract

Background: Atypical porcine pestivirus (APPV) is a neurotropic virus associated with congenital tremor type A-II. A few experimental studies also indicate an association between APPV and splay leg. The overarching aim of the present study was to provide insights into the virome, local cytokine response, and histology of the CNS in piglets with signs of congenital tremor or splay leg.

Results: Characterization of the cytokine profile and virome of the brain in piglets with signs of congenital tremor revealed an APPV-associated upregulation of Stimulator of interferon genes (STING). The upregulation of STING was associated with an increased expression of the gene encoding IFN-α but no differential expression was recorded for the genes encoding CXCL8, IFN-β, IFN-γ, IL-1β, IL-6, or IL-10. No viral agents or cytokine upregulation could be detected in the spinal cord of piglets with signs of splay leg or in the brain of piglets without an APPV-infection. The histopathological examination showed no lesions in the CNS that could be attributed to the APPV-infection, as no difference between sick and healthy piglets could be seen.

Conclusion: The results from this study provide evidence of an APPV-induced antiviral cytokine response but found no lesions related to the infection nor any support for a common causative agent.

Keywords: Congenital tremor, Type A-II, Atypical porcine pestivirus, Splay legs, Sweden, Pigs, Piglets, Pathology, Immunology, Virome, IFN-α, STING

Background

Atypical porcine pestivirus (APPV) is a newly detected neurotropic virus [1] that has been associated with congenital tremor type A-II through both experimental and natural infections [2–5]. The virus has also been associated with splay leg by experimental infections (Arruda et al., 2016, de Groof et al., 2016). However, APPV has never been demonstrated in naturally occurring cases of splay leg [6].

Both congenital tremor and splay leg are congenital disorders and their clinical signs are present directly from birth. Piglets born with congenital tremor present a varying degree of action tremor and, occasionally, ataxia. The lethality of congenital tremor ranges from around 10% to 30% at affected farms, mainly due to malnutrition or crushing by the sow [2, 3, 7]. The condition is reversible and piglets that survive until weaning often recovers completely [5, 8]. Based on the presumed aetiology and the pathoanatomical lesions, five known A-types of congenital tremor, characterized by hypomyelination and

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vacuolization in the white matter of the brain and one B-type associated with no microscopical lesions and no known causative agent are described [9–12].

Splay leg, spraddle leg syndrome, or porcine splay leg syndrome (PCS) is characterized by an impaired adduction of the hind limbs, and sometimes also the front legs [13]. The at-farm prevalence of splay leg ranges from 1–8% globally [14, 15], with a lethality up to 50% due to hypoglycaemia or mother overlying, crushing [16]. The impairment of the limbs is attributed to a hypomyelination of the spinal cord and the nerves innervating the affected muscles and a myofibrillar deficiency described as myofibrillar hypoplasia, an impaired muscular differentiation [13, 17, 18]. These histological findings are not pathognomonic for splay legs since they are subtle and can be seen in healthy neonatal piglets [16, 19]. Several risk factors, both extrinsic and intrinsic, are associated with splay leg *i.e.* hereditary factors [20], insufficient intrauterine nutrition [21, 22], induction of parturition [23], genetics [24, 25], and viral infections [2, 3, 26].

The virus that causes congenital tremor type A-II, APPV, belongs to the species *Pestivirus K* of the genus *Pestivirus* and the family *Flaviviridae* [1, 27]. It is a single-stranded RNA virus with a polyprotein consisting of 12 proteins: C (capsid protein), E^{rns}, E1, E2 (envelope proteins), and non-structural proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [1, 27, 28]. The virus is known to induce a humoral immune response in affected pigs which is characterized by antibodies against the E2 and E^{rns} -proteins [29, 30] but, to date, nothing is known about the local inflammatory response in the CNS.

Although congenital tremor and splay leg are syndromes that have been known for decades and have a global distribution [5, 13, 15, 31, 32] knowledge of the pathology and causative agent(s) of these syndromes is still scarce. The aim of the present study was therefore to provide a more comprehensive description of natural cases of congenital tremor type A-II and splay leg, respectively, by high-throughput sequencing to characterize the virome of the brain tissue in piglets with signs of congenital tremor and the spinal cord tissue in piglets with signs of splay leg. In addition, histological examinations of the CNS were performed together with screenings for local cytokine production in the brain tissue of piglets suffering from congenital tremor and in the spinal cord tissue of piglets suffering from splay leg.

Results

Sequencing

Comparison of the results generated from Kraken 2 run against the non-redundant protein database (*nr*) to the DIAMOND run against the non-redundant protein database (*nr*) revealed in general similar results. However, the Kraken 2 run against the nucleotide non-redundant (*nt*) NCBI database was the only run that generated APPV hits in the piglets where the virus previously was demonstrated by PCR (Stenberg et al., 2020a). Hence, the Kraken 2 runs against the nucleotide non-redundant (*nt*) NCBI database were deemed to produce the most reliable results for this dataset and were therefore chosen for the taxonomic classification. An additional file shows the specific details for each sample (see additional file 1).

Piglets with signs of congenital tremor (NovaSeq)

Metagenomic sequencing was performed on brain tissue originating from six piglets with clinical signs of congenital tremor. The sequencing was performed on the Illumina NovaSeq platform. Four piglets, PCR-positive for APPV-genome in the brain, and two piglets PCR-negative for APPV-genome [6] were selected.

After quality control and filtering, the sequencing generated between 5.71 and 72.67 million reads. Of these reads, on average 78% mapped against the *Sus scrofa* genome and were removed. After removal of the host genome, about 90% of the remaining reads could be taxonomically classified. A majority of the reads, approximately 80%, were of chordate origin, mainly *Sus scrofa*. About 6% of the reads were of microbial origin. Less than 1% of the reads were classified as viral. Specific alignment against the APPV-genome identified APPV-sequences in all the four PCR-positive samples but not in the two PCR-negative samples.

In the APPV-PCR positive piglets, the most abundant viral reads were reads classified as APPV. The sample with the highest number of viral reads had 140 reads classified as APPV or Pestivirus K. The reads classified by Kraken as APPV were extracted and blasted using BLASTn and generated only APPV hits. Considerably fewer reads classified as other viruses were detected. The second most common viral taxon detected was also identified in all samples. These were reads classified by Kraken as Human respovirus 1 with 16 reads, at the most, in one sample. The reads were blasted against the non-redundant database *nt*, using BLASTn and generated Human respovirus hits. Kraken also detected a few reads from one sample classified as HIV, but after blast validation, these turned out to be false positives.

In the APPV-PCR negative piglets, the total number of viral reads was lower than in the APPV-PCR positive piglets. Only one virus with more than 10 reads was detected; 17 reads of Human immunodeficiency virus 1 in one sample, were confirmed as false positives with BLAST.

At the contig level, more than 88% of the de novo assembled contigs in all samples were classified. The majority of the classified contigs were of chordate origin

and less than 1% of the contigs were viral. The only virus with more than 1 contig was APPV which rendered, at the most, 10 contigs in one sample. The contigs classified as APPV by Kraken were extracted and blasted with web BLASTn and generated APPV hits. No viral contigs were detected in the APPV-negative piglets.

Piglets with signs of splay leg (MiSeq and NovaSeq)

Metagenomic sequencing was performed on tissue originating from the thoracic part of the spinal cord from seven piglets with signs of splay leg; tissue from six of the piglets was subjected to sequencing on an Illumina MiSeq platform and tissue from one piglet was subjected to sequencing on an Illumina NovaSeq platform. All of these seven piglets were PCR-negative for APPV-genome in the spinal cord [6].

The MiSeq sequencing generated between 0.94 and 78.10 million reads per sample after quality control and filtering. Of these reads, about 80% of the reads could be mapped against the host genome and were removed. Of the remaining reads, approximately 80% could be taxonomically classified. About 35% of the reads were of chordate origin, mainly *Sus scrofa*. The majority of the classified reads, approximately 45%, were of microbial origin but less than 0.1% were classified as viral. Alignment against the APPV-genome did not generate any hits.

In this dataset, there was no detection of true viral reads. There were reads classified as viral by Kraken, but once the reads were extracted and submitted for a BLAST search only reads classified as Equine infectious anemia virus generated hits in BLAST. Virus classified as Equine infectious anemia virus is assumed to be a reagent contaminant commonly detected in metagenomic datasets [33]. The other reads classified as viral reads by Kraken did not generate viral hits when blasted but mainly hits from the pig genome.

At the contig level, more than 90% of the de novo assembled contigs were classified. The majority of the contigs were of chordate origin and less than 0.1% of the contigs were of viral origin. Viral contigs were detected in five of the six samples, however in very low numbers. The most abundant viral finding were contigs classified as Edafosvirus sp. and generated three contigs in one sample. When these contigs were blasted they aligned to the pig genome. One contig classified as Equine infectious anaemia virus was detected in all samples, when these contigs were submitted to BLAST they aligned to the reagent contaminant Equine infectious anaemia virus genome.

After filtering and quality control, the NovaSeq sequencing generated 2.07 millionreads. Approximately 79% of the reads could be mapped against the *Sus scrofa* genome and were removed. After host mapping was performed, 99.6% of the reads could be taxonomically classified. Of these were 44% of chordate origin and 53% were classified as microbial. Less than 0.01% of the reads were of viral origin. Reads from two viruses were classified by Kraken: Orthohepevirus A (Hepatitis E) and Influenza A virus but when blasted these reads did not generate viral hits. No viral contigs were generated from this dataset. Specific alignment against the APPV-genome did not generate any hits.

Healthy piglets (MiSeq)

Metagenomic sequencing was performed on brain tissue originating from eight clinically healthy piglets, PCR-negative for APPV-genome, on an Illumina MiSeq platform.

For seven of the eight samples, the sequencing generated between 8.13 and 50.64 million reads per sample after quality control and filtering. One sample (sample 18) was underrepresented during sequencing and generated only 758.77 thousand reads. In all samples approximately 88% of the reads mapped against the *Sus scrofa* genome and were removed. Of the remaining reads, about 60% could be taxonomically classified. A majority of the reads were of microbial origin, most of them classified as bacterial. Less than 0.01% of the reads were classified as viral. Specific alignment against the APPV-genome did not generate any hits.

In all samples, a few reads were classified as viral by Kraken but after blast validation, all of them turned out to be from the *Sus scrofa* genome. This could stem from misclassification, due to how Kraken's algorithm works, or from the fact that some sequences in the databases are wrongly annotated.

At the contig level, more than 30% of the de novo assembled contigs were classified. A majority of the contigs were of microbial origin and less the 1% of the contigs were classified as viral. A few viral contigs were detected in all samples, but after blast validation almost all of them turned out to be from the *Sus scrofa* genome. One contig classified as Equine infectious anemia virus was detected in all samples and could be blast validated as Equine infectious anemia viru and thus most likely a reagent contaminant [33]

Pathology

Gross findings

The necropsy of the healthy piglets, the piglets with signs of congenital tremor, and the piglets with signs of splay leg revealed no gross lesions. The results of the necropsy are described in detail by Stenberg et al. (2020a).

Histopathologic evaluation of brain and spinal cord

Scattered within the white matter at all levels of spinal cord and brain were a few multifocal, small vacuoles with variable shape and size. The vacuoles were randomly distributed with no associated cell reaction. In the brain, vacuoles were most common in cerebellum. In the spinal cord, vacuoles were more commonly seen, as compared to the brain. Vacuoles were present in equal numbers in the brain and spinal cord in healthy piglets and in piglets with signs of congenital tremor or splay legs. (Fig. 1, A & B).

The cerebellum and spinal cord from three piglets with high virus load and from one control piglet, were further investigated for myelin loss and changes and/or loss of nissl substances using luxol fast blue stain and cresyl violet stain. There were no pathologic findings consistent with a decreased amount of myelin or loss of nissl substance within the neurons, the staining intensity of the tissue being similar in the sick and healthy piglets. (Fig. 1, C & D).

Transmission electron microscopy

Tissue from the cerebellum from two piglets (one with signs of congenital tremor and one clinically unaffected) were subjected to transmission electron microscopic examination.

The piglet with signs of congenital tremor showed degenerated mitochondria, a mild separation of myelin lamellae, and small sporadic vacuoles in the neuropil. The degenerated mitochondria, characterized by swelling and loss of crista structure, were mainly present in the oligodendrocytes but also occasionally seen in the axons (Fig. 2).

In the cerebellar tissue of the clinically unaffected piglet, minor ultrastructural changes such as mild separation of myelin lamellae were detected.

Cytokine analyses

The expression of cytokine genes associated to viral infections was studied in the brain tissue from piglets with signs of congenital tremor, positive for APPV ($n=13$)

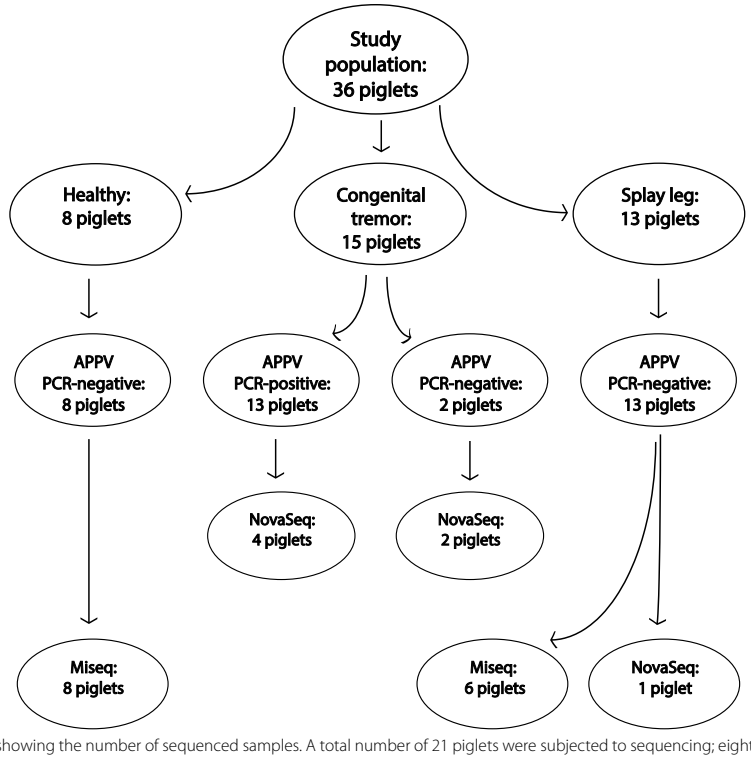

Fig. 1 Flow chart showing the number of sequenced samples. A total number of 21 piglets were subjected to sequencing; eight healthy, six with signs of congenital tremor, and seven piglets with signs of splay leg. The piglets were selected to represent all sampled farms and litters

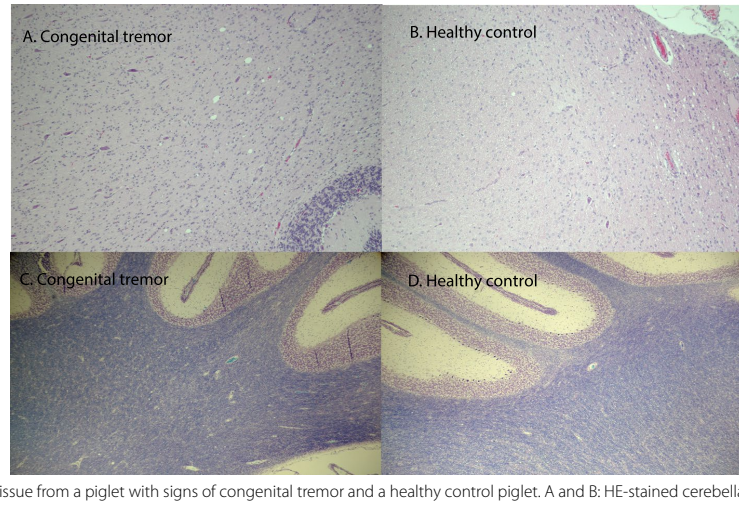

Fig. 2 Cerebellar tissue from a piglet with signs of congenital tremor and a healthy control piglet. A and B: HE-stained cerebellar tissue from a piglet with signs of congenital tremor and a healthy control piglet. A few multifocal, small vacuoles with variable shapes and sizes can be seen in equal numbers in the sick and healthy piglet. Scale bar: 200 µm. C and D: Cerebellar tissue from a piglet with signs of congenital tremor and a healthy control piglet stained with luxol fast blue stain and cresyl violet stain. No myelin loss or loss of nissl substances can be seen. Scale bar: 500 µm

and compared to the cytokine gene expression in samples from five of the clinically healthy piglets. For comparison, the cytokine genes that were found to be upregulated in these APPV-infected piglets were also analysed in spinal cord tissue from piglets with signs of splay leg ($n=12$) and in the brain tissue from the two APPV-negative piglets displaying clinical signs of congenital tremor.

Cytokine profile of APPV-infected brains

The expression of selected cytokine genes (CXCL8, IFN−α, IFN-β, IFN-γ, IL-1β, IL-6, IL-10, and STING) in brain tissue of APPV-positive pigs with signs of congenital tremor was related to the gene expression in brain tissue from five of the healthy piglets. Of these, the gene encoding STING was significantly up-regulated compared to the healthy control group. An up-regulation of IFN-α was also indicated but not statistically significant. (Fig. 3). However, a bivariate analysis found a significant correlation between the expression of STING and IFN-α ($r=0.824$, $p<0.0001$). The gene encoding IFITM3 was detected in all brain samples, but not differentially expressed (FC: 1.1 ± 1.9) compared to the healthy control group (FC: 1.4 ± 1.5). Expression of the genes encoding CXCL8, IFN-β, IFN-γ, IL-1β, IL-6, and IL-10 were not detected in the brain tissue of APPV-positive piglets despite that these genes were detected, although at high Cq values, in the brain tissue of the healthy piglets.

Significant upregulation, $p<0.05$, of STING encoding gene in piglets PCR-positive for APPV and with signs of congenital tremor compared to the healthy control piglets. An up-regulation of IFN-α is indicated, however not statistically significant.

For the spinal cord tissue from piglets with signs of splay leg, the three genes that were detected in the APPV-positive piglets; STING, IFN-α, and IFITM3 were analysed but no expression of these genes were found. Thus, the presence of APPV appears to induce a STING-associated cytokine profile in porcine brain tissue. Supporting this, STING and IFN-α were not differentially expressed in the brains of the two CT piglets negative for APPV.

Discussion

The present study aimed to characterize the virome, histopathology and cytokine profile of the CNS in piglets with signs of congenital tremor or splay legs. Comparison of these data from piglets with the two diseases indicated that congenital tremor and splay legs have different pathogenesis and, likely, different causative agents, despite sharing some clinical traits. In the present study, APPV was only detected in the brain tissue of piglets with signs of congenital tremor, and no virus or cytokine upregulation was detected in the spinal cord from piglets with splay legs, indicating that APPV is not the causative agent for this syndrome in natural infections.

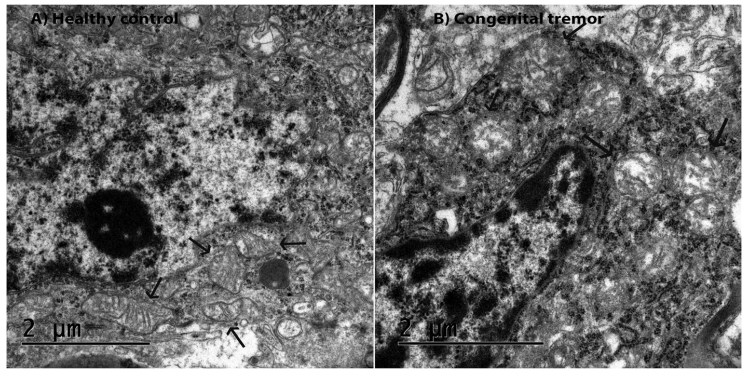

Fig. 3 Oligodendrocytes from cerebellar tissue visualised by transmission electron microscopy. Transmission electron microscopic examination of cerebellar tissue from a piglet with signs of congenital tremor and a healthy control piglet. Mitochondria are marked with black arrows. In the healthy piglet, mitochondria are normal in size with a prominent crista. Numerous degenerated mitochondria, swollen with loss of crista, can be seen in the oligodendrocyte of the sick piglet

Given that most piglets with congenital tremor type A-II recover fully from their signs and clear the APPV-infection [29] there has to be a functional immune mechanism restricting the viral replication. In accordance, transcriptional analysis of brain tissue from piglets with signs of congenital tremor and a concurrent APPV-infection revealed an upregulation of the gene encoding STING, a transmembrane protein at the endoplasmic reticulum involved in the activation of type-I interferons [34]. Interestingly, upregulation of STING could be correlated with an increased expression of the gene encoding IFN-α, indicating that APPV elicits antiviral innate immune reactions in the piglet brain. This antiviral response can be directly associated with APPV, as this was the only viral finding in the metagenomic sequencing of the brain tissue. The concept of the APPV-associated cytokine profile is further supported by the lack of upregulation of these cytokines in piglets where APPV was not found.

Interestingly, the upregulation of STING in the APPV infected brain is similar to the immune response in the brain following infection with the Zika virus, which is, similar to APPV, a neurotropic, small, enveloped ssRNA + virus [35, 36]. Zika infection is known to induce upregulation of STING [37], activate anti-viral autophagy [37], and stimulate the local immune response to increase the production of type-I interferons, such as IFN-α, in the brain. Although STING mainly has been associated with the recognition of cytosolic DNA [34], these mechanisms make STING important to the restriction of both RNA and DNA virus replication [38–40].

Stimulation of type-I IFN genes through STING has to be carefully regulated since an excessive production or continuous occurrence of type-I IFNs, *e.g.,* IFN-α, not only generates an anti-viral state in the cells of the surrounding tissue but may trigger tissue damage, especially in the brain [41]. A recent study on the immune response towards herpes simplex virus type-1-infection in the CNS suggests that the cGAS/STING pathway in the brain has a regulatory effect to protect the tissue from damage due to high IFN type-I activity [42]. This regulatory "feed-back loop" will make type-I IFN producing immune cells, microglia, in particular, undergo STING-dependent apoptosis if the immune-stimulatory signals are too intense *e.g.,* if the viral load is high [42]. Such a protective mechanism may be one possible explanation for the lack of extensive histopathologic lesions such as hypomyelination, apoptosis, or nerve cell damage in the CNS of piglets with signs of congenital tremor, despite a high presence of APPV in the tissue. However, if this is due to STING-mediated regulation remains to be elucidated.

Commonly, congenital tremor type A-II has been associated with histopathological lesions such as vacuolization of the white matter and hypomyelination in the brain [4, 10, 43–45]. These types of lesions were, however, not seen in the brain or spinal cord of any of the Swedish piglets. Interestingly, the histopathological lesions previously described by others are similar to descriptions of artifacts due to delayed fixation of the tissue. These artifact vacuoles and myelin alterations are often particularly prominent within the white matter of the CNS [46, 47]. In addition, it should be noted that the studies where

severe lesions have been recorded lacks descriptions of the time from death to removal and fixation of the CNS tissue. Further, in these studies, the occurrence of non-APPV viral co-infections cannot be excluded. Reports of these lesions, therefore, need to be interpreted with caution. In the present study, measures were taken to avoid bias related to post-mortem autolysis. The pigs were brought alive to the Department of Pathology and euthanized immediately before necropsy and fixation of tissue. Thus, it can be speculated that the severity of the APPV-associated lesions in the CNS might not be as extensive as previously described. Nevertheless, the TEM examination in the present study revealed degenerated mitochondria, mainly in the myelin-producing oligodendrocytes, as well as mild myelin disruption in the cerebellum. This is indicative of a pathological process involving the myelin that might induce CNS-derived signs such as shaking.

The APPV associated upregulation of STING in the present study is further supported by the metagenomic sequencing results that ensure the absence of non-APPV viral co-infections. Overall, the metagenomic sequencing resulted in a low number of viral reads, both in the brain and spinal cord. The most obvious finding that emerged from the metagenomic analysis is that APPV could be detected in the brain tissue of APPV PCR-positive piglets with signs of congenital tremor. In these NovaSeq sequenced samples, reads classified as human respovirus-1 were also detected. Human respovirus causes a seasonal upper respiratory tract infection, common in humans [48] and the finding is probably caused by human contamination during library or sequencing preparation. Another probable contaminant was detected in the Miseq generated datasets, *i.e.,* in the datasets of the healthy piglets and the piglets with signs of splay leg; reads and contigs classified as Equine infectious anemia. The common discovery of reads and contigs classified as Equine infectious anemia virus in datasets produced by metagenomic next-generation sequencing has recently been attributed to a reagent contaminant, a novel reagent-associated lenti-like virus [33]. Phylogenetic analysis of this novel reagent-associated lenti-like virus shows that it is closely related to and clusters with several known sequences of Equine infectious anemia virus, making it a recurrent classification and alignment error in datasets [33].

No true viral hits could be detected in the APPV-PCR negative piglets with signs of congenital tremor, the piglets with signs of splay leg, or the healthy piglets. It can therefore be assumed that the sequencing depth was enough to detect a clinical infection as well as obvious contamination. However, it may be speculated that part of the full CNS-virome, such as phages or endogenous retroviruses, was lost during pre-preparation and library preparation. Almost all pigs carry porcine endogenous retroviruses incorporated in their chromosomes [49, 50] but these viruses will not be detected in datasets if precautions are taken to remove the host genome *i.e.,* pig genome, as done in the present study. To date, there are no published studies on the porcine CNS-virome available for comparisons with the results from the sequencing in the present study, since publications describing the porcine virome, with a few exceptions, mainly focus on organs relevant for xenotransplantation [49].

The outcome of the metagenomic and transcriptional analyses in the present study provides no evidence for a common causative agent or pathogenesis of splay leg and congenital tremor, although the diseases may have some common clinical traits. This result is contrary to previous studies *e.g.*, by Arruda et al. (2016) or de Groof et al. (2016), who suggested that congenital tremor and splay leg both can be induced by a transplacental infection with APPV. This discrepancy may be attributed to the fact that Arruda et al. (2016) and de Groof et al. (2016) conducted experimental infections, either by inoculation of APPV directly to the foetal amniotic vesicle or infection of the pregnant gilt by a combination of oral, subcutaneous, intramuscular, and intranasal inoculation. Likely, this is a more intense exposure to the APPV, as compared to a natural infection. Thus, it can be hypothesized that neurotropic APPV may induce several signs derived from interference in the CNS, such as an inability to control the extremities if the infection is excessive. However, natural infections with APPV have only been associated with signs of congenital tremor [6, 29, 51, 52].

The results from this study have increased the understanding of the local immune mechanisms in the APPV-infected brain and added to the knowledge of the ultrastructural changes in the brain of piglets with signs of congenital tremor. It also lays the groundwork for a further understanding of the CNS virome in sick and healthy piglets. Several questions, for example how the APPV-infection resolve and how permanent damage to the brain is prevented, still remain to be answered and further research on the topic is needed.

Conclusion

The aim of the present study was to provide a more comprehensive description of natural cases of congenital tremor type A-II and splay leg, respectively. An APPV-associated cytokine profile, characterized by an upregulation of the gene encoding STING has been identified, as well as a lack of histopathological lesions but the presence of ultrastructural changes in the brain of a piglet with congenital tremor. The study could not identify any viral agent in the spinal cord of piglets with signs of splay leg and the study provides no evidence for a common

causative agent or pathogenesis of splay leg and congenital tremor.

Material and method

Study population

The study included archived material from 36 new-born piglets; brain tissue from eight healthy control animals, spinal cord tissue from 13 piglets with signs of splay leg, and brain tissue from 15 piglets with signs of congenital tremor. The tissue originated from animals sampled in 2017/2018, as described by Stenberg et al. (2020a), and stored at − 80 °C. Brain tissue (gray matter from the cerebrum) was analysed from piglets withs signs of congenital tremor and tissue from the spinal cord (the thoracic part) was analysed from piglets with signs of splay leg. Different tissues were chosen for analysis since congenital tremor is associated with infections and lesions in the brain (Arruda et al., 2016, de Groof et al., 2016, Postel et al., 2016, Stenberg et al., 2020) whereas splay leg is associated with lesions and infections in the spinal cord (Papatsiros, 2012, Jeong et al., 2017, Schumacher et al., 2021).

The general condition of the piglets, sampling, and farm of origin as well as specific Cq-values and a phylogenetic analysis of the virus is described in detail in Stenberg et al., (2020a). The piglets with signs of congenital tremor originated from five farms and six litters. The piglets with signs of splay leg originated from four farms and eight litters. The healthy piglets originated from four farms and seven litters. The healthy piglets and the piglets with signs of splay leg were APPV-genome negative in the brain and spinal cord tissue, respectively, whereas 13 out of 15 piglets with signs of congenital tremor were APPV-genome positive in the brain tissue [6]. The two APPV-genome negative piglets with clinical signs of congenital tremor were littermates, and the only piglets sampled at that specific farm. In addition, two 5-day old piglets were sampled in January 2022, one with signs of congenital tremor, and one healthy control piglet from a litter containing only healthy piglets. Both piglets originated from a farm experiencing an outbreak of congenital tremor and were only included in the histological and transmission electron microscopy (TEM) analyses. All animal studies were approved by the ethical committee for animal experimentation of Uppsala 2017–02-10 (Dnr 5.8.10–00,431/2017) in accordance with the current European (directive 86/609/EEC) and the Swedish legislation (Djurskyddslag 2018:1192). All methods are reported in accordance with ARRIVE guidelines.

All piglets were transported to and euthanized at the pathological facility at the Swedish University of Agricultural Sciences in Uppsala. The piglets were sedated with an intramuscular injection of tiletamine and zolazepam (Zoletil®, Virbac, Carros, France) and euthanized by an intraperitoneal injection of pentobarbital (Allfatal vet. Apotek Produktion & Laboratorier AB, Malmö, Sweden). To ensure quick and correct handling of the piglets and to minimize the time from death to necropsy to a few minutes the piglets were sedated and euthanized one at a time.

High-throughput sequencing

RNA-extraction

The brain and the spinal cord samples were cryolyzed in ice-cold PBS using a Precellys tissue homogenizer (Bertin Corp. Rockville, MD, USA) and centrifuged for 10 min at 4 000 RCF in 4 °C. The supernatants were transferred to a spin filtrate column (0.45 µm) and centrifuged for 4 min at 4 000 RCF in 4 °C. The filtrates were treated with 2U of TURBO™ DNase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and DNase I (Invitrogen, Life Technologies, Carlsbad, CA, USA) and incubated for 30 min at 37 °C. Thereafter, a total volume of 200 µL was treated with 5 µL RNase Cocktail™ (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 5 min at room temperature.

RNA was extracted using a trizolphenol-chloroform protocol and cleaned using the GeneJET RNA kit (ThermoFisher Scientific, Waltham, MA, USA). During the RNA extraction, an "on-column" DNase digestion was performed using the RNase-Free DNase Set (QIAGEN GmbH, Hilden, Germany). The concentration of the purified RNA was quantified on a Qubit® 2.0 Fluorometer using the Qubit RNA HS assay kit (Thermo Fisher Scientific, Paisley, UK).

Library preparation and sequencing

Sequencing libraries were prepared in two batches, first for sequencing on a MiSeq instrument (Illumina, San Diego, CA, USA) at the National Veterinary Institute, Uppsala, Sweden, and later for sequencing on a NovaSeq 6000 system at the SNP&SEQ Technology Platform at SciLifeLab in Uppsala (Fig. 4).

MiSeq sequencing

In the MiSeq sequencing, the eight healthy piglets and six piglets with signs of splay leg were included. Sequencing libraries for the MiSeq run were prepared using the Trio RNA-Seq Library Preparation Kit (NuGEN Technologies, San Carlos, CA, USA). The library concentration was measured using a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Paisley, UK) and an Agilent High Sensitivity DNA Kit (2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). Based on the concentration, pooling and normalization to

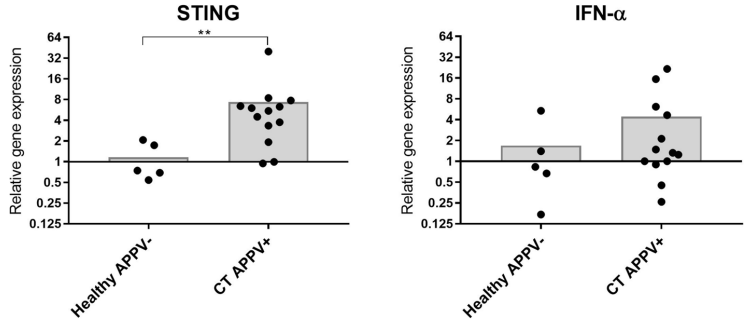

Fig. 4 Graph presenting the expression of STING and of IFN-α encoding genes. Significant upregulation, $p < 0.05$, of STING encoding gene in piglets PCR-positive for APPV and with signs of congenital tremor compared to the healthy control piglets. An up-regulation of IFN-α is indicated, however not statistically significant

2 nM were performed. The library pools were diluted in RNase-free water. Paired-end sequencing was performed with a MiSeq Reagent Kit v3 600 cycles on the MiSeq instrument (Illumina, San Diego, CA, USA) at the National Veterinary Institute, Uppsala, Sweden. The quality of the dataset was assessed using the FastQC software [53].

NovaSeq sequencing

In the NovaSeq sequencing, seven piglets with signs of congenital tremor, one from each sampled litter and the two APPV-genome negative piglets as well as one piglet with signs of splay leg were included. The sequencing libraries for NovaSeq were prepared using the Trio RNA-Seq Library Preparation Kit with the Custom AnyDeplete for targeted pig genome depletion (NuGEN Technologies, San Carlos, CA, USA). Library pooling and normalization of 5 ng DNA/µL were performed based on the concentration recorded by Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Paisley, UK) and TapeStation (Agilent, Santa Clara, CA, USA). RNase-free water was used for the library dilution.

Using the NovaSeq 6000 system, a paired-end 150 bp read length sequencing was performed using an SP flow cell and the v1 sequencing chemistry (Illumina, San Diego, CA, USA). A 1% spike-in with a sequencing library for the phage PhiX was included in the run. The FastQC software [53] was used to assess the quality of the produced dataset.

Assembly of sequence reads

To analyse the produced datasets a NextFlow pipeline was implemented. The pipeline included the following steps: quality control and trimming of the reads using FASTP version 0.19.5 [54], removal of the host reads by mapping on *Sus scrofa* genome using bowtie2 version 2.3.5.1 [55], and de novo assembly of the remaining reads using MEGAHIT version 1.2.9 [56]. Taxonomic classification of the reads was performed using Kraken 2 version 2.0.8-beta [57] against the nucleotide non-redundant (*nt*) NCBI database. For the taxonomic classification of the assembled contigs, two methods were used and compared: Kraken2 and DIAMOND version 0.9.24.125 [58]. Kraken2 was run against both the non-redundant protein database (*nr*) and the nucleotide non-redundant (*nt*) NCBI database, and DIAMOND was run against the *nr* protein database. The pipeline is fully available online at GitHub (https://github.com/jhayer/nf-metavir).

The resulting reports were visualised using Pavian [59]. Additionally, specific alignments against the APPV genome (NC_030653.1 Atypical porcine pestivirus 1 isolate Bavaria S5/9 polyprotein gene, complete cds) were performed using Bowtie2 version 2.3.5.1 [55].

Cytokine analysis

RNA isolation and cDNA synthesis

Isolation of RNA from brain tissue and spinal cord was performed as previously described by Stenberg et al. (2020a). The quantity and purity of the extracted RNA were measured by spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Montchanin, DE) and Agilent High Sensitivity DNA Kit (2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). Three of the eight healthy control piglets were excluded due to non-sufficient RNA concentration for the cDNA protocol. Synthesis of cDNA was performed using an input of 1.2 µg RNA per reaction (GoScript Reverse transcription system, Promega). To remove potential

contamination of genomic DNA, the RNA was treated with RQ1 RNAse-free DNAse (Promega, Madison, WI, USA), and a -RT control was run parallel to the RNA samples. All samples were diluted 1:5 in RNase-free water and stored at − 20 °C until use.

qPCR analysis

Expression of the genes encoding CXCL8, IFN-α, IFN-β, IFN-γ, IFITM3, IL-1β, IL-6, IL-10, and STING was estimated using previously published primer pairs and assay conditions [60]. All samples were run in duplicate reactions of 25 µL with 2 µL cDNA in 23 µL Quantitect SYBR Green PCR mix (Qiagen) on a CFX96 Touch PCR machine (Bio-Rad). The cycling protocol was initiated by a cycle of 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at the assay-specific annealing temperature, 30 s at 72 °C, ending with a melt curve analysis to verify the PCR product. To enable relative quantifications, primer pairs for five reference genes; GAPDH, HPRT, PPIA, RPL32, and YWHAZ were tested for their expression stability in a representative selection of porcine brain tissue using previously established assay conditions [60, 61]. Based on geNorm analysis (qBase^{PLUS}, Biogazelle), the genes for GAPDH (M = 1.32; CV = 0.76), HPRT (M = 1.17; CV = 0.42), and RPL32 (M = 1.06; CV = 0.32) were selected for normalization of data. For each cytokine gene, the Cq value was normalized to the geometric mean of the three reference genes. The relative quantity of each gene was calibrated to the mean of the healthy control pigs ($n = 5$). Genes with fold change values < 0.5 or > 2 were regarded as down- or up-regulated.

Statistical analysis

Statistical analysis was performed using the software Prism 7.0 (Graph-Pad). Differences in the expression of cytokine genes between the APPV negative healthy piglets and APPV positive piglets were calculated using an unpaired t-test on ΔΔCq values. To test for correlation in the expression of STING and IFN-α, a bivariate fit analysis was performed using the JMP® Pro 15.2.0. The model included the observations of the expression of STING and IFN-α from the thirteen APPV-genome positive piglets with signs of congenital tremor. For all tests, p-values below 0.05 were regarded as significant. When indicated, variability of gene expression data is reported as mean FC ± SD.

Pathology

At necropsy, the brain and spinal cord were immediately removed first to ensure a quick fixation, thereby reducing the risk for post mortem artifacts. The tissue samples were immediately fixed in 10% neutral buffered formalin. Eight sections were taken from the CNS, five from the brain and three from the spinal cord; globus pallidus, the parietal cortex, hippocampus, thalamus, mesencephalon, cerebellum, obex, post colliculus, and the cervical, thoracic-, and lumbar part of the spinal cord. All paraffin-embedded tissue was sectioned at 4 µm and mounted on slides that were routinely stained with haematoxylin and eosin for histologic review. In addition, the cerebellum and spinal cord from three piglets with the highest viral load and one control piglet were stained using luxol fast blue stain and cresyl violet stain.

Transmission electron microscopy

Two piglets, one with clinical signs of congenital tremor and one healthy control piglet, sampled in 2022, were selected for TEM. Tissue from the cerebellum were cut into cubes of 1 mm^3 before fixation.

Fixation and embedding

For transmission electron microscopy, the samples were fixed in 2.5% glutaraldehyde (Ted Pella INC, Redding, CA, USA) + 1% paraformaldehyde (Merck, Darmstadt, Germany) in PIPES (Merck, Darmstadt, Germany) pH 7.4, and stored at 4 °C until further processed. Samples were rinsed with 0.1 M phosphate buffer for 10 min prior to 1 h incubation in 1% osmium tetroxide (TAAB, Aldermaston, England) in 0.1 M phosphate buffer. After rinsing in 0.1 M phosphate buffer, samples were dehydrated by incubation in increasing concentrations of ethanol (50%, 70%, 95% and 99.9%) for 10 min each, followed by 5 min incubation in propylene oxide (TAAB, Aldermaston, England). The tissue samples were thereafter placed in a mixture of Epon Resin (Ted Pella INC, Redding, CA, USA) and propylene oxide (1:1) for 1 h, followed by 100% resin, and left over night. Subsequently, the samples were embedded in capsules in newly prepared Epon resin, left for 1–2 h, and then polymerized at 60 °C for 48 h.

Sectioning and contrasting

The specimens were cut into semi-thin Sects. (1–2 microns), stained in Toluidine Blue and examined by light microscopy. The blocks were trimmed and ultrathin Sects. (60- 70 nm) were cut in an EM UC7 Ultramicrotome (Leica, Stockholm, Sweden), and then placed on a grid. The sections were subsequently contrasted with 5% uranyl acetate and Reynold's lead citrate and visualized with Tecnai™ G2 Spirit BioTwin transmission electron microscope (Thermo Fisher/FEI, Hillsboro, Oregon, USA) at 80 kV with an ORIUS

SC200 CCD camera and Gatan Digital Micrograph software (both from Gatan Inc, Pleasanton, CA, USA).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-022-03443-w.

Additional file 1.

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Authors' contributions
Hedvig Stenberg: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing—original draft, writing—review & editing, visualization, project administration, and funding acquisition. Stina Hellman: conceptualization, methodology, validation, formal analysis, investigation, writing—review & editing, visualization, and funding acquisition. Lisa Lindström: writing—review & editing and investigation. Magdalena Jacobson: writing—review & editing and resources. Caroline Fossum: writing—review & editing, supervision. Juliette Hayer: methodology, software, validation, resources, data curation, writing—review & editing and supervision. Maja Malmberg: methodology, resources, writing—review & editing, supervision, project administration, and funding acquisition. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets generated and/or analysed during the current study are available in the PRJEB50949 repository at the European Nucleotide Archive. [https://www.ebi.ac.uk/ena/browser/view/PRJEB50949?show=reads, accession number: SAMEA13443979—SAMEA13444000].

Declarations

Ethics approval and consent to participate
All animal studies were performed in accordance with the current European (directive 86/609/EEC) and the Swedish legislation (Djurskyddslag 2018:1192). The study was approved by the ethical committee for animal experimentation of Uppsala 2017–02-10 (Dnr 5.8.10–00431/2017) and the owners of the herds gave informed consent prior to the start of the study. All methods are reported in accordance with ARRIVE guidelines.

Consent for publication
Not applicable.

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

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ORIGINAL ARTICLE

Atypical porcine pestivirus—A widespread virus in the Swedish wild boar population

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Abstract

The recently identified causative agent of congenital tremor in domestic piglets, atypical porcine pestivirus (APPV), was detected in serum from Swedish wild boar. A previous study from Sweden described APPV in domestic piglets suffering from congenital tremor, but the APPV situation in the wild boar population was unknown. In this study, 595 serum samples from wild boar originating from 13 counties in the south and central parts of Sweden, collected between 2000 and 2018, were analysed for the presence of the APPV-genome and for antibodies against the APPV-glycoprotein E^{rns}. The results revealed that APPV is highly abundant in the Swedish wild boar population; 12% (73/595) were APPV-genome positive in serum and 72% (433/595) of the tested wild boars displayed APPV-specific antibodies. The present study also shows that APPV has been present in the Swedish wild boar population since at least the year 2000. The viral sequences obtained from the wild boars were highly similar to those obtained from Swedish domestic pigs positive for APPV and suffering from congenital tremor, suggesting a viral exchange between wild boars and domestic pigs. The high proportion of viraemic and seropositive wild boar is indicative of wild boar being an important reservoir for APPV.

KEYWORDS
APPV, atypical porcine pestivirus, genome detection, reservoir host, serology, wild boar

1 | INTRODUCTION

Atypical porcine pestivirus (APPV) was discovered in healthy pigs in the United States by high-throughput sequencing in 2014 (Hause et al., 2015). It is a ssRNA+ virus of the family Flaviviridae. The APPV is classified as the species *pestivirus K* in the genus *Pestivirus* (Hause et al., 2015; Smith et al., 2017). The virus is genetically diverse and the genome varies both within and between countries (Postel, Meyer, Cagatay, et al., 2017; Sutton et al., 2019). It possesses three envelope proteins; E^{rns}, E1 and E2 (Hause et al., 2015). The protein E^{rns} induces a humoral immune response following an infection and has been used in previous studies to diagnose APPV in wild boars (Cagatay et al., 2018, 2019).

Shortly after its discovery in 2015, the presence of APPV was correlated with congenital tremor in newborn piglets (Arruda et al., 2016, Postel et al., 2016). Although experimental infection with a virus isolate is pending and the Koch's postulates have not been fulfilled yet,

experimental inoculation of APPV genome positive sample material can induce congenital tremor in piglets (Arruda et al., 2016; de Groof et al., 2016). Congenital tremor or *myoclonia congenita* is a congenital disease occurring in piglets characterized by tremor and ataxia (Stenberg et al., 2020b). Moreover, several studies have indicated that APPV might induce splay leg in addition to congenital tremor in newborn piglets (Arruda et al., 2016; de Groof et al., 2016; Stenberg et al., 2020b). A recent study revealed that APPV is also frequently found in Swedish piglets with signs of congenital tremor type A-II (Stenberg et al., 2020a).

Since its first detection in the United States of America, APPV has been detected in domestic pigs in Canada and South America (Dessureault et al., 2018; Hause et al., 2015; Possatti, De Oliveira, et al., 2018), Europe (Postel, Meyer, Cagatay, et al., 2017; Schwarz et al., 2017; Stenberg et al., 2020a), and Asia (Dessureault et al., 2018; Hause et al., 2015; Possatti, Headley, et al., 2018; Postel, Meyer, Cagatay, et al., 2017; Schwarz et al., 2017; Shen et al., 2018; Stenberg et al., 2020b; Yuan et al., 2017). Despite this global spread of APPV, the prevalence varies between geographic areas. In a study including 1460 serum samples from healthy domestic pigs from Germany, Italy, Serbia, Great Britain, Switzerland, China, and Taiwan, the APPV-genome prevalence was 8.9% in total, in Europe varying from 2.3% in Great Britain to 17.5% in Italy (Postel, Meyer, Cagatay, et al., 2017). In the same study, the seroprevalence of APPV-specific antibodies was 60%; 27% (394/1460) of the pigs displayed high antibody levels whereas 33% (486/1460) had intermediate antibody levels. A study on the seroprevalence of APPV-specific antibodies on German pig farms revealed that approximately 37% of the 62 farms tested in 2018 were antibody-positive (Michelitsch et al., 2019).

There is a very small body of literature that is concerned with wild boar and APPV, and no studies have been conducted in the Nordic countries. A high APPV seroprevalence has been demonstrated in wild boars in Germany (52%, 237/456) and Serbia (67%, 10/15) (Cagatay et al., 2019). Interestingly, studies on the prevalence of APPV-genome in wild boar present a large discrepancy in the proportion of viraemic animals in different countries. A low occurrence of APPV-PCR positive wild boars was found in South Korea (18/2297), Spain (1/437) and Italy (3/430), but a higher proportion of APPV-PCR positive wild boars was detected in Germany (87/465) (Cagatay et al., 2018; Choe et al., 2020; Colom-Cadena et al., 2018; Sozzi et al., 2019). These studies reveal that the prevalence of APPV differs between geographic regions and that the wild boar population in some areas might play an important role as a reservoir for APPV. Overall, these studies highlight the need for further research.

In Sweden, the wild boar population has increased substantially in the last 40 years from about 100 in 1980 to an estimate of 300 000 individuals in 2019 (Naturvårdsverket, 2020). In the 1970s several wild boars escaped or were released from enclosures in the south and central parts of Sweden. Escapees quickly established a permanent population and have since spread predominantly in the southern parts of Sweden. Now, due to global warming, the wild boars are also expanding north along the Swedish east coast, though in the northern counties the wild boar population is still sparse (Naturvårdsverket, 2020). Although the Swedish wild boar is free-ranging, they cluster in the central and southern parts of Sweden, especially in the counties of Skåne and Södermanland, the same counties where wild boars first became established (Naturvårdsverket, 2020).

Wild boar is an important reservoir for pathogens that may infect domestic pigs, as well as a reservoir for pathogens of zoonotic potential. Therefore, the Swedish National Veterinary Institute performs yearly surveillance of specific pathogens in wild boars. At present, Sweden has a disease-free status for several epizootic pathogens, for example, classical and African swine fever virus, porcine reproductive and respiratory syndrome virus (PRRSV), and Pseudorabies virus (National Veterinary Institute, 2020). But a recent study shows that other viruses such as porcine parvovirus and porcine circovirus type 2 are highly abundant in Swedish wild boars with a seroprevalence of 78% and 99%, respectively (Malmsten et al., 2018). Despite the increasing wild boar population and the wild boars' importance as a reservoir for significant pathogens in other countries, the awareness of viral infections in the Swedish wild boar population is mainly limited to those included in the national surveillance programs.

This is the first study on APPV in the wild boar population in Sweden and any of the Nordic countries. The primary aim was therefore to determine whether APPV is present in the Swedish wild boar population by testing serum samples for the presence of APPV genome and APPV-specific antibodies, respectively. Any detected APPV genomes were then used to evaluate the genetic relatedness of APPV in Swedish wild boar and domestic pigs.

2 | MATERIALS AND METHODS

2.1 | Serum samples

Serum samples from 595 Swedish wild boars were collected between 2000 and 2018 and tested for APPV-specific antibodies against the APPV envelope glycoprotein E^{rns} and for the presence of the APPV-genome. All sampled wild boars originated from the central and southern parts of Sweden (Figure 1).

The majority of the samples ($n = 464$) were collected by hunters from wild boars after culling using sterile blood sampling tubes. The blood was then sent to the National Veterinary Institute, Uppsala, Sweden for separation of serum and storage at −80°C in the bio-bank. These serum samples were collected for the wild boar surveillance program, run by National Veterinary Institute and financed by the Swedish Board of Agricultures. The serum samples from 2000 ($n = 19$) lacked information on sampling location, and samples from 2000 to 2001 ($n = 13$) lacked information both on the sampling date and sampling location.

An additional 131 serum samples from female wild boars were collected in the years 2013–2014 by Malmsten et al. (2017). Here, blood was collected from the jugular vein or the thoracic cavity using sterile blood sampling tubes. The blood was clarified by centrifugation and the serum was separated and stored in a −20°C freezer. The reproductive status (gilt or sow) of 117 of the 131 female wild boars was recorded (Table 1).

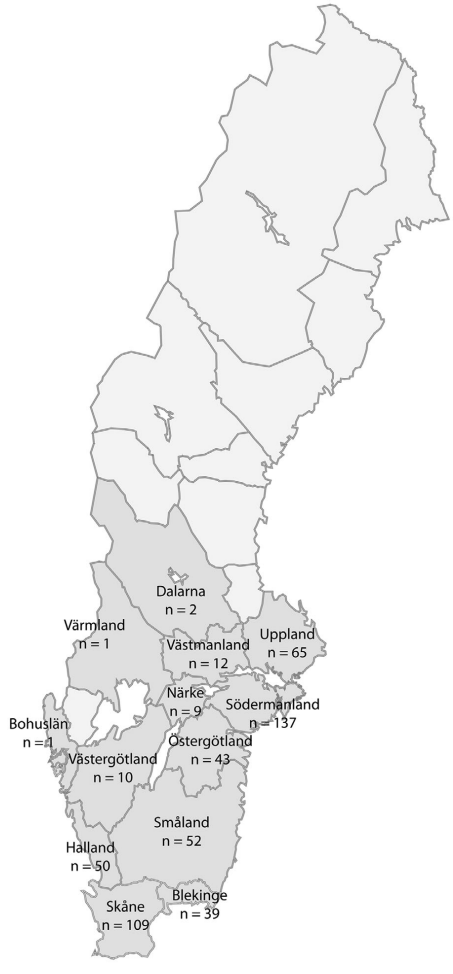

FIGURE 1 A map of Sweden highlighting the counties where wild boars were sampled. '*N*' represents sample size for the specific county. The counties with the densest wild boar populations, Skåne and Södermanland, are also the counties where the highest number of wild boars have been sampled

2.2 | Indirect APPV-specific ELISA

For antibody detection, an indirect APPV-specific enzyme-linked immunosorbent assay (ELISA) based on the glycoprotein E^{rns} (Postel, Meyer, Petrov, et al. , 2017) was used. Briefly, the APPV E^{rns} antigen was expressed in *Leishmania tarentolae*, purified by fast protein liquid chromatography (FPLC) and coated onto ELISA plates. Blocking was performed with PBS containing 0.05% Tween-20 and 4% skim milk powder at room temperature for 2 h. The E^{rns} ELISA assay was performed by incubating the serum samples diluted 1:25 in PBS containing 0.05% Tween-20 at 37°C for 1 h. Specific binding of antibodies was detected by peroxidase-labelled rabbit anti porcine IgG (A5670, Sigma-Aldrich, MO, USA, diluted 1:35,000) and 3,3′,5,5t'Tetramethylbenzidine (TMB, Sigma-Aldrich, MO, USA) according to the manufacturer's protocol. Porcine serum, confirmed positive for APPV E^{rns} -specific antibodies by Western blot, was used as positive control.

Initially, 88 randomly selected serum samples from the years 2000 ($n = 9$), 2001 ($n = 5$), 2002 ($n = 4$), 2005 ($n = 18$), 2009 ($n = 18$), 2017 ($n = 18$), and 2018 ($n = 16$) were tested in duplicates to assess the reliability of the assay. After establishing that the ELISA provided consistent results for the duplicates, the remaining serum samples were run as singles. To ensure a reliable inter-assay comparability, the serological values are presented as 'S/P-values' (sample/positive control). To facilitate the between-study comparability, the serological values were classified into low ($S/P \leq 0.5$), intermediate ($0.5 < S/P < 1.0$), or high reactivity ($S/P \geq 1.0$) as described previously (Cagatay et al., 2018, 2019; Grahofer et al., 2020; Postel, Meyer, Petrov, et al. , 2017). Consistent with the other studies using the same ELISA, the cut-off threshold for a positive serum sample was set to $S/P > 0.5$, consequently wild boars with a low serum reactivity ($S/P \leq 0.5$) were regarded as serologically negative.

TABLE 1 The number of samples collected each year[a] and, if known, whether the sampled wild boar was a gilt or a sow

Year	Number of samples	Unknown gender (*n*)	Gilts (*n*)	Sows (*n*)
2000	19	19	–	–
2000/2001	13	13	–	–
2001	23	23	–	–
2002	43	43	–	–
2005	100	100	–	–
2009	100	100	–	–
2013	116	14	41	61
2014	15	–	10	5
2017	84	84	–	–
2018	82	82	–	–
Total	595	472	51	66

[a]The total number of samples is 595, of them, 472 were of unknown gender, 51 were recorded as gilts and, 66 were recorded as sows.

2.3 | RNA purification and real-time RT-PCR

For the detection of APPV genome in the serum samples, the 595 samples were first tested in pools of five containing 40 μl of each serum sample. To ensure the efficacy of the RNA isolation and the RT-PCR, in vitro-transcribed EGFP-RNA was added to each serum pool as internal control (Hoffmann et al., 2005). RNA extraction was performed according to an accredited protocol of the EU and OIE Reference Laboratory for Classical Swine Fever using the KingFisher™ Duo Prime Purification System (ThermoFisher Scientific, Waltham, USA) and the IndiMag® Pathogen Kit (Indical Bioscience, Leipzig, Germany) as

TABLE 2 Primers used from detection of the APPV genome

Primer name	Sequence (5′ → 3′)	Region	Use	Reference
APPV-7001fw	GTGTCCAATTTTTGGGGCGTGTC	NS4B	Real-time RT-PCR	This study
APPV-7125rev	GCRACYACCACTGCTGATTCCAT	NS4B	Real-time RT-PCR	This study
APPV_5030-fw	CCCAGGCAATACCTCACAAC	NS3	Conventional RT-PCR and sequencing	(Cagatay et al., 2018)
APPV_5835-rev	TTCCTCTGGCCCTGTTCTTC	NS3	Conventional RT-PCR and sequencing	(Cagatay et al., 2018)

recommended by the manufacturers. Purified RNA pools were directly submitted to RT-PCR or stored at −80°C.

Given the growing number of available APPV sequences, a multiple sequence alignment was performed to identify a conserved region suitable for detection of genetically distinct APPV variants by real-time PCR. Using representative sequences belonging to APPV clade I that comprises all European sequences known so far (Folgueiras-González et al., 2020), a highly conserved region in the NS4B encoding sequence was identified and used to design the primer pair, APPV-7001fw / APPV-7125rev (Table 2). Validation of the new primer pair using serial dilutions of genomic RNA obtained from a cell culture infected with the German APPV isolate L277 (GenBank MF157291) as well as diagnostic samples sent to the Institute for Virology in Hannover demonstrated a sensitivity comparable to the previously used primer pair APPV-5587fw / APPV-5703rev (Cagatay et al., 2018). Furthermore, the primer pair showed less unspecific amplifications, making it particularly suitable for testing of wild boar samples. Different annealing temperatures were tested to optimize PCR conditions.

For the detection of APPV-genomes, a one-step SYBR-Green real-time RT-PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Feldkirchen, Germany) using the QuantiTect SYBR® Green RT-PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Each reaction contained 12.5 μl RT-PCR mastermix, 10 pmol of each primer, 0.25 μl RT-Mix, 5.25 μl nuclease-free water and 5 μl RNA amplicons. The thermal profile was applied as follows: 50°C for 30 min, 95°C for 15 min and 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s, followed by a melting curve analysis (from 65°C to 95°C, 0.5°C increment). PCR amplicons with a melting temperature (T_m) between 75.5 and 78.5°C were regarded as APPV positive.

Subsequently, individual samples from APPV positive pools were tested. For most samples a limited amount of serum was left (40–70 μl). When the serum volume was limited, 150 μl phosphate-buffered saline (PBS) was added prior to RNA preparation. RNA purification and real-time RT-PCR were conducted as described above. Selected samples were subjected to agarose gel electrophoresis to confirm specificity of the amplification.

2.4 | Conventional RT-PCR and nucleotide sequencing

Among the APPV-genome positive samples, differences in the melting curves could be observed, indicating genetical diversity. To obtain representative wild boar APPV sequences, 14 sera resulting in real-time RT-PCR amplicons with different melting temperatures were selected for generation of a larger genome fragment located in the NS3 encoding region. The selected samples included sera from Västmanland, Södermanland and Blekinge since from these counties, APPV sequences from domestic pigs are available for comparison and phylogenetic analysis (Stenberg et al., 2020a).

To generate amplicons for sequencing, a conventional two-step RT-PCR was performed. For cDNA-synthesis, M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, USA) and random hexamer primers (Invitrogen, Carlsbad, USA) were used. Amplification of an 806 bp fragment within the NS3 encoding region was obtained using the primers APPV_5030-fw and APPV_5835-rev (Cagatay et al., 2018) and the DreamTaq™ Hot Start Green PCR Master Mix (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Each reaction contained 45 μl master mix, 20 pmol of each primer and 3 μl cDNA. The following thermal profile was applied: 95°C for 2 min, 40 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min, and final extension at 72°C for 5 min. The PCR amplicons were purified using the GeneJET PCR Purification Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Sanger sequencing was conducted by LGC Genomics (Berlin, Germany) applying both primers that were used to generate the amplicons. All sequences generated in this study are available on ENA, Accession numbers: ERS6287711-15.

2.5 | Phylogenetic analysis

A phylogenetic analysis was performed based on the NS3 region of APPV. Five sequences of APPV genome fragments from Swedish wild boar were obtained from the PCR amplicons (806 nt).

All available APPV NS3 sequences were mined from GenBank and aligned using MUSCLE (Edgar, 2004) implemented in Genious prime (https://www.geneious.com). Maximum likelihood trees were estimated implementing the best nucleotide substitution model in PhyML (Guindon et al., 2010). Support values were calculated using aBayes, a bayesian approach for rapid support estimation (Anisimova et al., 2011). The tree was analysed using the TempEst software for temporal signals and 'clocklikeness' of the molecular phylogenies (Rambaut et al., 2016).

2.6 | Statistical analysis

A statistician was consulted, and the statistical analysis was performed using the JMP® Pro 15.2.0 software. Statistics are reported

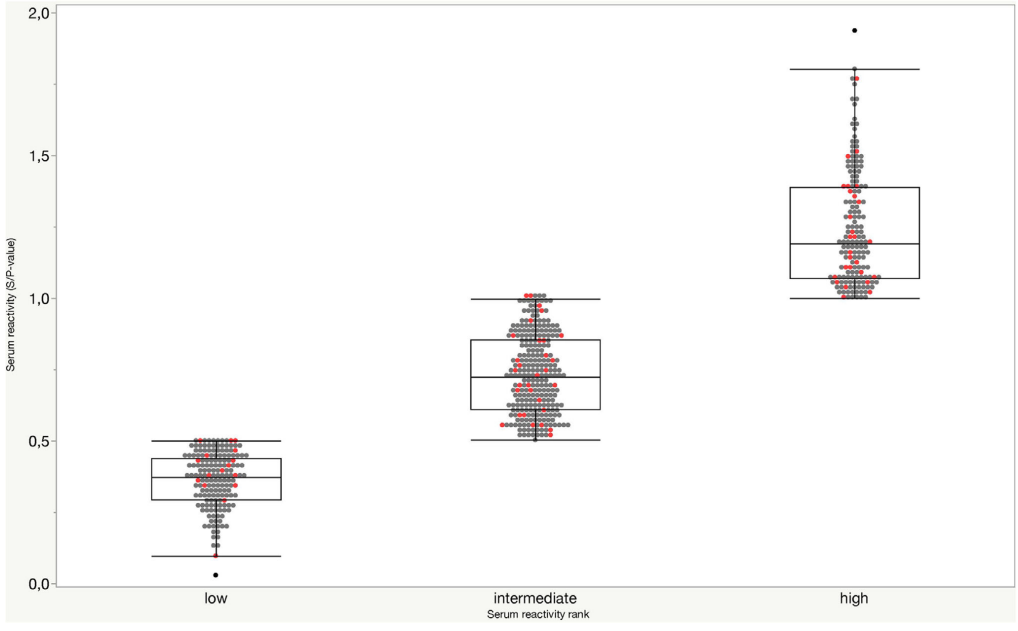

FIGURE 2 Boxplots showing the individual S/P-value (sample/positive control) for each of the 595 wild boars and how they allocate into the three subgroups of serum reactivity; low, intermediate, and high. The cut-off for a sero-positive sample is a S/P-value > 0.5. Each dot represents an individual wild boar and the red dots represent the APPV PCR-positive individuals. Low ($S/P \leq 0.5$), 28%, $n = 169$, mean = 0.36, APPV PCR-positive = 9% (16/169). Intermediate ($0.5 < S/P < 1.0$), 44%, $n = 264$, mean = 0.73, APPV PCR-positive = 11% (30/264). High ($S/P \geq 1.0$) 27%, $n = 162$, mean = 1.25. APPV PCR-positive = 17% (27/162))

as means and percentages. The dataset was analysed for differences in antibody reactivity between gilts and sows, annual differences, seasonality, and differences between the counties. To analyse the seasonality the months were subdivided in to: winter (December–February), spring (March–May), summer (June–August), and autumn (September–November).

The serum reactivity (S/P-value) was analysed by a general linear model including sampling year, county and season as fixed factors. This model included 395 of the total 595 observations. The excluded 200 observations could not be included in the model due to a lack of metadata concerning one or several of the fixed factors. The reproductive status (gilt, sow) was excluded as a factor from the general linear model since it could only include 116 of the total 595 observations, and all of these samples were collected in the years 2013–2014 in four counties (Blekinge $n = 35$, Skåne $n = 23$, Södermanland $n = 57$ and, Uppland $n = 1$). Hence, reproductive status was included in a separate general linear model. Pairwise comparisons for each factor, that is, year, county and season, were made by Tukey's honest significant difference test with the significance level set to $\alpha = 0.05$.

No statistical test was performed on the PCR-data because the number of positive samples was limited.

3 | RESULTS

3.1 | APPV in serum from Swedish wild boar

Sera from 595 wild boars sampled in Sweden in years between 2000 and 2018 were tested for the presence of APPV-genome and for the presence of APPV-specific antibodies. Overall, 72% (433/595) of the wild boars displayed APPV specific antibodies with an intermediate to high reactivity, and 12% (73/595) of the wild boars were APPV-genome positive (Figure 2). The proportion of wild boar with an intermediate serum reactivity was relatively consistent throughout the years, whereas the proportion of animals with high serum reactivity fluctuated. The largest proportion of APPV-genome positive wild boars was found among wild boars with a high antibody reactivity.

The general linear model described in Material and Methods was fitted to the data with the serum reactivity (S/P-value) as the response and year, county and season as the explanatory variable. Both year and county were found to be significant predictors of the serum reactivity (S/P-value). The season, however, was not a significant predictor. The model had an R^2 of 0.27 ($F(19, 394) = 7.385$, $p < .0001$). The model included 395 of the total 595 observations but all the 595 serum

samples from the years 2000 to 2018 are presented in the boxplots shown in Figures 2 and 3.

3.2 | Serum reactivity and APPV-genome detection in years between 2000 and 2018

The statistical model found the year to be a significant predictor of the serum reactivity ($F(6, 394) = 7.589$, $p < .0001$). The serum reactivity had the appearance of a sine wave, peaking in the years 2013–2014 (Figure 3, Panel A).

The pairwise comparisons made by Tukey's honest significant difference test showed that there was significantly higher serum reactivity ($n = 104$, S/P-mean = 0.977; $p < .05$) in 2013 than in 2002 ($n = 42$, S/P-mean = 0.489) and 2005 ($n = 95$, S/P-mean = 0.612).

The APPV-genome was detected by PCR in the wild boar serum from each year with a varying rate of PCR-positive animals. When comparing the years with > 80 observations, that is, the years 2005, 2009, 2013, 2017, and 2018, the proportion of APPV-genome positive animals seemed to increase over the years, varying from 6% (6/100) in the year 2005 to 24% (20/82) in the year 2018 (Figure 3, Panel A).

3.3 | Serum reactivity and APPV-genome detection in the sampled counties

The statistical model found the county to be a significant predictor of the serum reactivity ($F(10, 394) = 3.636$, $p = .0001$). It included 395 of the total 530 observations; the results from the 530 samples where the county was recorded are presented in the boxplot (Figure 3, Panel B).

The pairwise comparisons made by Tukey's honest significant difference test showed that Södermanland, a county situated in the central parts of Sweden, stands out with higher serum reactivity among the wild boars compared to samples from other counties. This serum reactivity ($n = 94$, S/P-mean value = 0.97) was significantly different ($p < .05$) from that in Halland ($n = 32$, S/P-mean = 0.58), Skåne ($n = 101$, S/P-mean = 0.7) and Blekinge ($n = 39$, S/P-mean = 0.77).

The APPV-genome was detected by PCR in serum from wild boars sampled in: Halland (6%, $n = 3/50$), Skåne (13%, $n = 14/109$), Småland (13%, $n = 7/52$), Södermanland (12%, $n = 16/137$), Uppland (9%, $n = 6/65$), Västergötland (40%, $n = 4/10$), Västmanland (8%, $n = 1/12$), and Östergötland (7%, $n = 3/43$) (Figure 3, Panel B).

3.4 | Serum reactivity and APPV-genome detection during summer, autumn, winter and spring

The statistical model showed that the season is not a significant predictor of the serum reactivity ($F(3,394) = 0.669$), $p = .573$). It included 395 of the total 530 observations; the results from the 399 samples where season was recorded are presented in the boxplot (Figure 3, Panel C).

The APPV-genome was detected by PCR with a varying prevalence in the wild boar sera from each season. Autumn (September–November) 13% (17/133). Winter (December–February) 10% (13/125). Spring (March–May) 5% (4/77). Summer (June–August) 3% (2/69) (Figure 3, Panel C).

3.5 | Serum reactivity and APPV-genome detection in female wild boars

The reproductive status of 117 female wild boars originating from four counties (Blekinge $n = 35$, Skåne $n = 23$, Södermanland $n = 57$ and, Uppland $n = 1$) was recorded, out of the total number of sampled wild boar (595). A general linear model was fitted with the serum reactivity (S/P-values) as the response, and reproductive status, year and county as the explanatory variable. The model showed that the county is a significant predictor of the serum reactivity in the female wild boar dataset ($F(8, 107) = 5.303$, $p < .0001$). The pairwise comparisons made by Tukey's honest significant difference test showed that the wild boars in Södermanland had a higher serum reactivity than the wild boars in Blekinge ($p < .05$). No statistically significant difference in the serum reactivity between gilts and sows was detected. However, there was a tendency to a lower antibody reactivity in the serum from sows compared to gilts (Figure 3, Panel D). The APPV-genome was detected by PCR in serum from both gilts and sows. The APPV-genome detection rate was 10% in gilts (5/51) and 8% in sows (5/66) (Figure 3, Panel D).

3.6 | Phylogenetic analysis

Conventional RT-PCR of 14 selected sera originating from different Swedish counties resulted in five amplicons suitable for sequencing. These were three samples from Södermanland and one from Blekinge, counties from which APPV sequences obtained from domestic pigs have been determined previously (Stenberg et al., 2020a). Additionally, it was possible to determine an APPV sequence from Uppland, from where no genetic information about APPV circulating in domestic pigs has been available before. Two distinct clusters of APPV in Swedish wild boar were detected in the phylogenetic analysis (Figure 4). The largest number of sequences were those falling into Cluster I, where all sequences from Sweden (both those generated in this study and sequences from domestic pigs described previously) fell into one discrete clade. This clade is largely dominated by sequences from Asia. A single Swedish wild boar sequence fell into Cluster II, which is largely dominated by sequences from European pigs and wild boars. The sequences from Sweden did not cluster into a single clade, and the sequence in cluster II generated from this study was more closely related to a sequence from Wild boars in Italy than to any sequences generated from pigs in Sweden. We were unable to make a definite estimate of the introduction date of these viruses into Sweden due to limitations in the temporal signal of the available data but the TempEst analysis put the introduction time of APPV to Sweden > 100 years ago.

4 | DISCUSSION

This is the first study on APPV genome- and seroprevalence in Swedish wild boar. The selection of archived samples from the national biobank

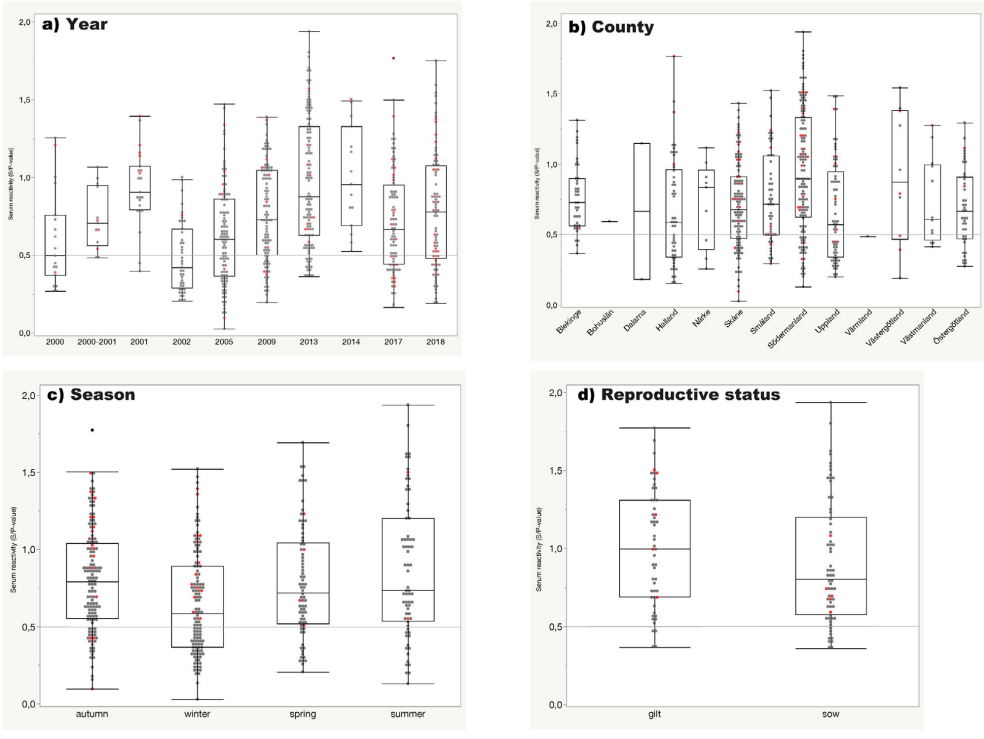

FIGURE 3 Serum reactivity and genome detection rate in Swedish wild boars from years between 2000 and 2018. Boxplot A). Distribution of the antibody reactivity or S/P-values (sample/positive control) in 595 serum samples from the year 2000 to 2018. The cut-off for a sero-positive sample is a S/P-value > 0.5, marked with a grey line in the figure. Each dot represents one individual wild boar and the red dots represents the APPV PCR-positive individuals. There is a weak trend to increasingserum reactivity (S/P-values) from 2000 to 2018. The serum reactivity has the appearance of a light wave-like motion, gradually increasing from 2000 to 2018, peaking in 2013. Year 2000, $n = 19$, S/P-mean = 0.601, Std dev. = 0.309, APPV PCR-positive = 11% (2/19). Year 2000–2001, $n = 13$, S/P-mean = 0.733, Std dev. = 0.197, APPV PCR-positive = 23% (3/13). Year 2001, $n = 23$, S/P-mean = 0.933, Std dev. = 0.245, APPV PCR-positive = 26% (6/23). Year 2002, $n = 43$, S/P-mean = 0.483, Std dev. = 0.224, APPV PCR-positive = 2% (1/43). Year 2005, $n = 100$, S/P-mean = 0.622, Std dev. = 0.318, APPV PCR-positive = 6% (6/100). Year 2009, $n = 100$, S/P-mean = 0.765, Std dev. = 0.308, APPV PCR-positive = 8% (8/100). Year 2013, $n = 115$, S/P-mean = 0.977, Std dev. = 0.410, APPV PCR-positive = 8% (9/116). Year 2014, $n = 15$, S/P-mean = 0.998409, Std dev. = 0.330, APPV PCR-positive = 7% (1/15). Year 2017, $n = 84$, S/P-mean = 0.720, Std dev. = 0.332 APPV PCR-positive = 20% (17/84). Year 2018, $n = 82$, S/P-mean = 0.789, Std dev. = 0.376, APPV PCR-positive = 24% (20/82).) Boxplot B). Serum reactivity and genome detection rate in 530 wild boars from the different counties. Mean and individual S/P-values. The cut-off for a sero-positive sample is a S/P-value > 0.5, marked with a grey line in the figure. Each dot represents one individual wild boar and the red dots are the APPV PCR-positive individuals. Blekinge: $n = 39$, S/P-mean = 0.77, Std dev = 0.24, APPV PCR-positive = 3% (1/39). Bohuslän: $n = 1$, APPV PCR-positive = 0. Dalarna: $n = 2$, APPV PCR-positive = 0. Halland: $n = 50$, S/P-mean = 0.66, Std dev = 0.38, APPV PCR-positive = 6% (3/50). Närke: $n = 9$, S/P-mean = 0.72, Std dev = 0.31, APPV PCR-positive = 0. Skåne: $n = 109$, S/P-mean = 0.71, Std dev = 0.31, APPV PCR-positive = 13% (14/109). Småland: $n = 52$, S/P-mean = 0.76, Std dev = 0.33, APPV PCR-positive = 13% (7/52). Södermanland: $n = 137$, S/P-mean = 0.95, Std dev. = 0.44, APPV PCR-positive = 12% (16/137). Uppland: $n = 65$, S/P-mean = 0.66, Std dev = 0.36, APPV PCR-positive = 9% (6/65). Värmland: $n = 1$, APPV PCR-positive = 0. Västergötland: $n = 10$, S/P-mean = 0.92, Std dev = 0.49, APPV PCR-positive = 40% (4/10). Västmanland: $n = 12$, S/P-mean = 0.74, Std dev = 0.31, APPV PCR-positive = 8% (1/12). Östergötland: $n = 43$, S/P-mean = 0.69, Std dev = 0.28, APPV PCR-positive = 7% (3/43).) Boxplot C). Serum reactivity and genome detection rate in 399 wild boars during the different seasons. Mean and individual S/P-values. The cut-off for a sero-positive sample is a S/P-value > 0.5, marked with a grey line in the figure. Each dot represents one individual wild boar and the red dots are the APPV PCR-positive individuals. Summer (June–August): $n = 69$, S/P-Mean: 0.87, Std dev = 0.446, APPV PCR-positive = 3% (2/69). Autumn (September–November): $n = 133$, S/P-Mean: 0.81, Std dev = 0.336, APPV PCR-positive = 13% (17/133). Winter (December–February): $n = 125$, S/P-Mean 0.65, Std dev = 0.332, APPV PCR-positive = 1% (13/123). Spring (March–May): $n = 77$, S/P-Mean: 0.79, Std dev = 0.363, APPV PCR-positive = 5% (4/77).) Boxplot D). Serum reactivity and genome detection rate in gilts and sows. Mean and individual S/P-values for 117 female wild boars. The cut-off for a sero-positive sample is a S/P-value > 0.5, marked with a grey line in the figure. Each dot represents one individual wild boar and the red dots are the APPV PCR-positive individuals. No significant difference was found in the serum reactivity between gilts and sows. Gilts: $n = 51$, S/P-mean = 1.012, Std dev = 0.370, APPV PCR-positive = 10% (5/51). Sows: $n = 66$, S/P-mean = 0.908, Std dev = 0.398, APPV PCR-positive = 8% (5/66).)

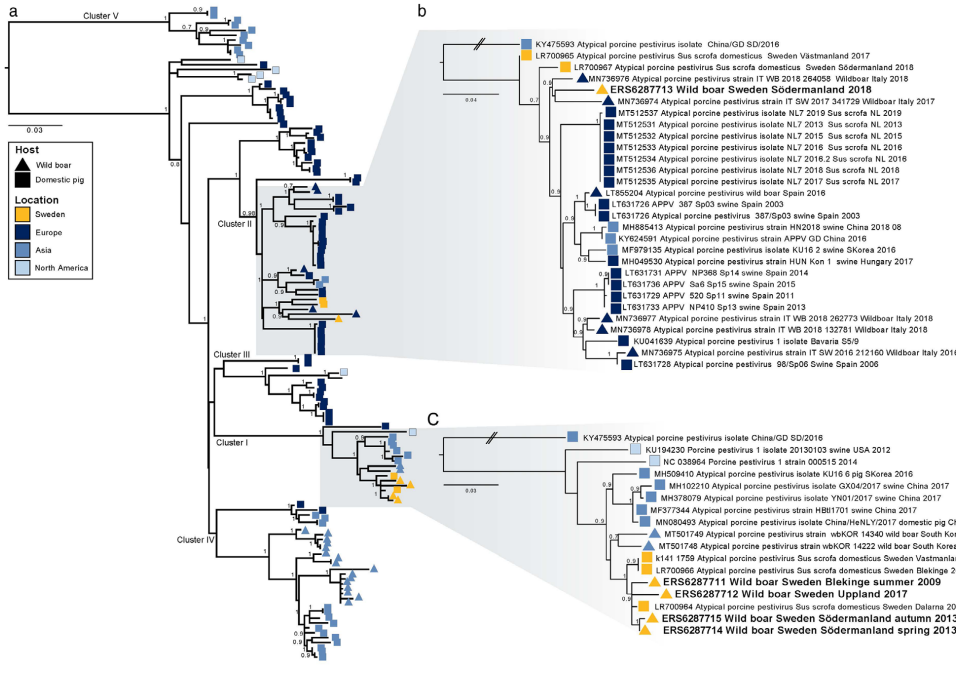

FIGURE 4 Phylogenetic Tree. Maximum likelihood tree of the NS3 region of Atypical Porcine Pestivirus. (a) Phylogeny of all NS3 sequences in GenBank. Cluster information from Gatto et al. (2019). Clade 5 was selected as the outgroup based on Folgueiras-González et al. (2020) and Gatto et al. (2019). (b,c) Expansion of select clusters to illustrate genetic relationship of sequences generated in this study. Cluster trees were rooted against KY475593, a sequence in Cluster V. Tips are coloured based on host and geographic location. Scale bar indicates number of substitutions per site. Bayesian support values (as calculated using aBayes; Guindon et al., 2010) are indicated on relevant nodes. Sequences names honour those in GenBank, such that a number of sequences are labelled 'Porcine Pestivirus 1' rather than 'Atypical Porcine Pestivirus.' Sequences generated in this study are in bold

provided an opportunity to advance the understanding of APPV in the Swedish wild boar population. The analyses revealed changes in the APPV-antibody and -genome prevalence during the past two decades as well as a difference in the occurrence of APPV between counties. Furthermore, an opportunity was obtained to genetically characterize the APPV isolates circulating in the Swedish wild boar population and to determine their genetic relatedness to APPV-sequences from Swedish domestic pigs suffering from congenital tremor.

Since APPV was only recently discovered, knowledge of the natural transmission, immunity and antibody kinetics is limited. A previous study evaluating the humoral immune response induced after infection with APPV showed that E^{rns}-specific antibodies were declining in a majority of the studied pigs after about 160 days whereas the E2-specific antibody levels did not decline significantly over the same period of time (Cagatay et al., 2019). Because of the more transient immune response to E^{rns} than to the E2 protein, the former was chosen to follow the dynamics of APPV infection in the Swedish wild boar population over time using an E^{rns} specific ELISA. Furthermore, because the APPV genome is highly variable, a new primer pair targeting a conserved sequence in the APPVs' NS4B encoding region was designed to ensure a high detection rate. Using these methods, 12% of the wild boars (73/595) were PCR-positive for APPV genome in serum and 72% (433/595) displayed APPV specific antibodies, indicating that APPV is abundant among wild boars in Sweden.

The high seroprevalence is in line with the results from another study where the APPV antibody prevalence in wild boar was determined using the same indirect APPV-specific ELISA (Cagatay et al., 2018). Hence, the results obtained from Germany and Serbia, where 52% of the tested wild boars had APPV specific antibodies, are comparable to the present Swedish results. Interestingly, a study from Switzerland also using the same indirect APPV-specific ELISA presented an occurrence of APPV antibodies of 93% in a closed APPV endemic herd of domestic pigs (Grahofer et al., 2020), suggesting that APPV has the potential to cause an extensive herd immunity.

The overall APPV genome detection rate of 12% in Swedish wild boar is also similar to the findings in German wild boar (18%) (Cagatay et al., 2018). But the proportion APPV-genome positive wild boars in the Swedish and German population are considerably higher

compared to South Korea, Spain and Italy, where the genome detection rate is < 1% (Cagatay et al., 2018; Choe et al., 2020; Colom-Cadena et al., 2018; Sozzi et al., 2019). Although the studies are not completely comparable, since primer pairs targeting different part of the genome has been used, they clearly demonstrate that the proportion of viraemic wild boars varies between countries. The cause of the variation in the APPV-genome detection rate between studies and countries is not known but viral spread in the wild boar population can be attributed to several factors differing between countries such as the population density, climate, fencing, agriculture, foresting and, hunting (Bertelloni et al., 2020; Malmsten et al., 2018; Morelle et al., 2020; Petit et al., 2020).

Based on the outcomes from this study, it is evident that APPV is common and has been present in the Swedish wild boar population since at least the year 2000. The seroprevalence in the years between 2000 and 2018 showed a wave-like pattern, similar to the epidemiology of other viral diseases (Stegmaier et al., 2020) (Figure 3, Panel A). In the current study, the year 2013 stands out with significantly higher antibody reactivity than the other years. The high antibody reactivity caused by an extensive spread of APPV is most likely attributed to the exceptionally mild winter of the years 2012/2013 (Wern, 2015). A mild winter would have led to a higher survival rate of piglets and consequently more yearlings in 2013, increasing the spread of pathogens (Podgórski et al., 2018). The high antibody reactivity detected in wild boar sera from the year 2013 might also be confounded by the high proportion of wild boars collected in Södermanland during the years 2013/2014.

The serum reactivity (S/P-values) of wild boars sampled in Södermanland was significantly higher than in wild boars sampled in Halland, Skåne and Blekinge, indicating a higher circulation of APPV in wild boars in Södermanland than in the other counties. The county of Södermanland is located in the central parts of Sweden, whereas, Halland, Skåne and, Blekinge are counties bordering each other in the south of Sweden (Figure 1). Interestingly, the proportion of APPV-genome positive wild boars in Södermanland and Skåne were very similar, 12% and 13% respectively, whereas the proportion of genome positive wild boars in Blekinge and Halland was lower, 3% and 6% respectively. The high genome detection rate in the wild boar population of Södermanland and Skåne was somewhat expected, since Södermanland and Skåne were the first counties in Sweden to be colonized by wild boar in the 1970s and have the densest wild boar populations in Sweden today (Naturvårdsverket, 2020).

Despite the limited number of samples, there is a trend to an increase in the proportion of APPV-genome positive animals over the years. The increasing genome detection rate and seroprevalence of APPV-specific antibodies from 2000 to 2018 could be attributed to the natural degradation of RNA and antibodies in serum during storage, but there are other more plausible explanations. The number of wild boars in Sweden has increased massively in the last decades, causing denser populations and more frequent interactions between groups. The increased wild boar population has also led to an increased interest in hunting but also in supplementary feeding (Naturvårdsverket, 2020). Hunting, as well as supplementary feeding, increases mobility and interactions between groups of wild boars and is known to escalate the spread of viruses within a wild boar population (Morelle et al., 2020). This human intervention may partly explain the higher proportion of viraemic wild boars during autumn and winter than during summer and spring but it also implicates transmission of APPV as being the most intense during the mating-season in autumn.

Horizontal as well as vertical transmission of APPV infection between domestic pigs is indeed very efficient (Cagatay et al., 2019) and pigs may shed virus in saliva, faeces, urine and semen for several months after infection, making mating a possible route of infection with APPV (Arruda et al., 2016; de Groof et al., 2016). The idea of APPV spread via semen as one possible route of infection is supported by a study on domestic pigs in which young sows presented with the highest E^{rns} antibody reactivity, and this reactivity decreased with time (Grahofer et al., 2020). If it is assumed that wild boar has the same shedding and spreading pattern as domestic pigs, it could be speculated that wild boar sows, which have been mated, should have a higher antibody reactivity than non-mated gilts. Nonetheless, no significant difference in antibody reactivity between Swedish wild boar gilts and sows was detected. There was, however, a weak trend of slightly higher antibody reactivity in gilts than in sows. This trend that could be due to the fact that younger animals (0.5–2 year) have more interaction with other wild boars, both within and between groups, making them more likely to contract infectious diseases (Podgórski et al., 2018).

At present, there are no reports of signs of congenital tremor in wild boar piglets but it cannot be excluded that APPV infection in wild boar is associated with congenital tremor. It seems plausible however, that it would be hard to find wild boar piglets with signs of congenital tremor since the sows keep newborn wild boar piglets hidden. In domestic pigs, however, there are regular reports of outbreaks of piglets born with signs of congenital tremor (Stenberg et al., 2020a). The routes of introduction of APPV into a closed swine herd is not clear but wild boars could be speculated to be a reservoir. The present study reveals that sequences from wild boar and domestics pigs from the same area are clustering together, indicating a transmission of APPV between domestic pigs and wild boars, as described for other viruses (Meng et al., 2009).

The phylogenetic analysis also revealed two clusters of APPV in Sweden, reflecting the diversity thus far described in Swedish pigs (Stenberg et al., 2020a). The clustering, where the major part of the Swedish sequences from wild boar and domestic pigs fell into one cluster (Cluster I), suggests a single introduction into Sweden and subsequent continuous circulation. Since wild boars were introduced to the Swedish fauna in the 1970s, it can be speculated that APPV was first introduced and circulated in the domestic pig population and then transmitted to the wild boar population but that wild boars today serve as the main reservoir for APPV. Although the TempEst analysis could not conclude on a set time of introduction of APPV to Sweden, it suggests that the virus has been circulating in Sweden for > 100 years. This is plausible since congenital tremor type A-II was described in Swedish domestic pigs in the 1950s (Larsson, 1955; Stenberg et al., 2020a).

Further, the clustering illuminated the need for further research on APPV in wild boar. The APPV belonging to the previously reported

phylogenetic cluster I is largely dominated by sequences from Asia, either suggesting hidden diversity in Europe, or representing the movement of pigs or wild boars from Asia to Scandinavia through anthropogenic assistance. Since the import of live pigs to Sweden is, and has been, very limited, an anthropogenic introduction of APPV to Sweden from Asia through live pigs is unlikely (Svenska djurbönders smittskyddskontroll, 2018; Swedish Board of Agriculture, 2021). The sequence generated from this study in cluster II was more closely related to sequences from wild boars in Italy than to any sequences generated from domestic pigs in Sweden. The close similarity between APPV from Swedish wild boar and APPV genomes detected in wild boar from Italy and South Korea demonstrates the knowledge gap concerning the APPV epidemiology in wild boar. Genetic characterization of APPV from wild boar originating from different countries should be performed to establish reliable epidemiological links. In general, epidemiology of viral pathogens circulating in wild animal species is still poorly understood and should be more strongly in the focus of future research.

5 | CONCLUSION

APPV was found to be highly abundant in the Swedish wild boar population. Of the 595 sampled wild boars, 12% (73/595) were viraemic and 72% (433/595) displayed APPV-specific antibodies. This study proves that APPV has been present in the Swedish wild boar population since at least the year 2000. The high proportion of viraemic and seropositive wild boar and the genetical closeness of APPV in wild boar and domestic pigs is indicative of wild boar being an important reservoir for APPV and calls for further research.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Hedvig Stenberg, Elena Leveringhaus, Alexander Postel and Maja Malmberg contributed to the conception and design of the study. Hedvig Stenberg analysed the data and drafted the manuscript. Elena Leveringhaus carried out the lab work. Anna Malmsten and Anne-Marie Dalin collected the serum samples from the female wild boars. All authors have approved the submitted version of the manuscript.

DATA AVAILABILITY STATEMENT

Sequence data have been submitted to the EMBL databases under accession number ERS6287711-15.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The animal samples used in this study were archived sera that had been collected by hunters in years between 2000 and 2018 as part of a national Swedish surveillance programme or collected post mortem at abattoirs for other studies. No ethical permit was required for collecting or using these samples.

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Congenital tremor and splay leg are neurological diseases regularly seen in new-born piglets in Sweden. The cause of the clinical signs is unknown, but impaired nerve function due to myelin damage is a theory. In this thesis, atypical porcine pestivirus was identified in piglets with signs of congenital tremor, Swedish wild boars, and semen intended for artificial insemination. In addition, the tissue-resident CNS virome and boar semen microbiome, including the virome, were studied.

Hedvig Stenberg received her graduate education at the Department of Biomedical Science and Veterinary Public Health. Her undergraduate degree in veterinary medicine was obtained at the Swedish University of Agricultural Sciences, Uppsala, Sweden.

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