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Metagenomic, Viral and Host Genetic Analyses of Congenital Tremor in Pigs

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METAGENOMIC, VIRAL AND HOST GENETIC ANALYSES OF CONGENITAL TREMOR IN PIGS

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METAGENOMIC VIRAL AND HOST GENETICS ANALYSES OF CONGENITAL TREMOR IN PIGS

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The *Pestivirus* genus contains several viral species having a major impact on the livestock species. While there were only four major pestiviral species for a time, recent metagenomic sequencing approaches identified additional species, such as atypical porcine pestivirus (APPV). Congenital tremor was first identified almost a hundred years ago and since still has an impact on swine health. There are two main types of congenital tremor, type A and B, with type A congenital tremor further subcategorized based on causative agent. Until recently, type A-II congenital tremor did not have a known cause. Recent viral sequencing of affected samples, including our research, revealed APPV to be the predominant viral species in congenital tremor piglets, providing evidence of a causative agent for type A-II congenital tremor.

Various strains of APPV have been identified across the globe. Through sequence comparison, it was found most of the viral APPV genome is not highly conserved. This can lead to misdiagnosis or false-negative results due to variations in the APPV genome sequence when testing of congenital tremor samples is based on qPCR assays. While degenerate primers are an option to combat this, the high degree of variation across the strains and rapid evolution of the viral genome will eventually lead to qPCR assays not recognizing all strains. Whole sequence comparison of full-length APPV genomes demonstrated the 5’ untranslated region (5’UTR) is highly conserved (85%) between 20

worldwide species. This high degree of conservation makes the 5'UTR an ideal candidate region for a universal qPCR assay to detect any strains of APPV.

The role of host genetics in disease susceptibility and severity have been documented for several swine viruses. The swine leukocyte antigen class II complex (*SLAII*) is involved in the antigen presentation during viral infection. The highly polymorphic *DQB1* gene in this region was partially sequenced to provide haplotype profiling to a group of sows subjected to an APPV-based exposure leading to litters with various degrees of congenital tremor. Relationships between *DQB1* haplotypes and incidence of congenital tremor and the rate of pre-weaning mortality were investigated. Future more detailed research will need to better quantify the role of host genetics in APPV susceptibility and disease progression.

CHAPTER 1: LITERATURE REVIEW

Pestiviruses are a genus in the family *Flaviviridae*. While for a long time there were only four recognized species that mainly infected pigs and ruminants, recent sequencing technology is shedding light on additional members which infect additional hosts such as bats and rodents. With the addition of new species, the impact on host health, livestock production, and the economy has increased. Pestiviruses such as classical swine fever virus (CSFV), atypical porcine pestivirus (APPV), border disease virus (BDV), and bovine viral diarrhea virus (BVDV) can infect livestock leading to a substantial loss if the animals are unable to make a recovery. While in some cases vaccination can help prevent these viral diseases, host genetic factors could also play a role in the susceptibility of hosts to these viruses. If there is a strong genetic component, farmers can breed for these traits in hopes of not losing profits due to disease, increase the genetic resistance of their population, and reducing animal welfare.

PESTIVIRUS

Structure

Pestivirus, a genus in the family *Flaviviridae,* is represented by single-stranded, positive-sense RNA viruses. These viruses are enveloped and with a size between 40-60 nanometers in diameter (Bruschke et al., 1997; Becher & Thiel, 1999; Lin et al., 2004; Tautz et al., 2015; Wang et al., 2015; Riedel et al., 2017; Braun et al., 2019). Until recently, there were four recognized species in the *Pestivirus* genus: classical swine fever virus (CSFV), bovine viral diarrhea virus-1 (BVDV-1), BVDV-2, and border disease virus (BDV). Lately, novel pestiviruses have been identified infecting various species. Most pestiviruses can be classified into either infecting swine or ruminants, with some of

the novel species also infecting bats, rats, and nematodes (Becher & Thiel, 1999; Lazar et al., 2003; Hause et al., 2015; Tautz et al., 2015; Beer et al., 2017; Postel et al., 2016; Blome et al., 2017; Riedel et al., 2017; Dessureault et al., 2018; Mósena et al., 2018; Shen et al., 2018; Braun et al., 2019; Cagatay et al., 2019; Kaufmann et al., 2019; Michelitsch et al., 2019; Pan et al., 2019).

Within this group, there are two biotypes: cytopathic and noncytopathic. Noncytopathic is identified in most isolates as it is the only type that can establish a persistent infection as an adaptive immune response is absent. It also does not induce cell death. Cytopathic pestiviruses, which can induce cell death, have an increased RNA replication which allows for a buildup of viral RNA, triggering an adaptive immune response and eliminating the virus (Tautz et al., 2015). The ability to establish a persistent infection is one of the main reasons pestiviruses can negatively impact livestock (El Omari et al., 2013; Wang et al., 2014; Tautz et al., 2015; Postel et al., 2016; Pan et al., 2019). Pestiviruses can cross the placenta and infect fetuses. However, since pig and ruminant placentas do not allow for the transfer of maternal antibodies, a mother's immune response cannot protect the fetal infection. Therefore, the time of infection during gestation is key in determining the severity of a disease and the ability of a fetus to mount an immune response. Infection of fetuses within approximately the first 40 days of gestation results in death, due to the lack of immune system in fetuses. However, if the fetal immune systems begin to develop after this point this could lead to persistent infections. Animals that are persistently infected lack an adaptive immune response; thus viral antibodies are absent. The adaptive immune response is either inhibited by pestiviruses or the virus develops ways to evade it (Tautz et al., 2015).

Pestiviruses genomes encode a single polypeptide that is post-translationally modified to produce 13 proteins (four structural, eight non-structural, one heterodimer; Figure 1.1). Flanking either side of open-reading frame are the 5'- and 3'- untranslated regions (UTRs) (Becher & Thiel., 1999; Lin et al., 2004; Tautz et al., 2015; Zhang et al., 2018; Guo et al., 2020). Within the 5'-UTR, there is a stable stem-loop structure that is involved with translation initiation and replication in studies of BVDV. The stable stem-loop is the most conserved region, being required for the initiation of transcription, while the remainder of the 5'-UTR is more variable. However, pestiviruses are dependent on the host's translational machinery. While the host RNA has a 5'-cap involved in the start of the translation, pestiviruses lack this characteristic and contain an internal ribosomal entry site (IRES) which ensures the host translation machinery begins at the correct start codon. This feature belongs to the family of IRES that also includes the human hepatitis C virus (HCV), which suggests that pestiviruses are good models for studying human viral disease (El Omari et al., 2013; Tautz et al., 2015). The other difference between eukaryotic RNA and pestiviruses is in the 3'-UTR with the poly-(A) tail missing from pestiviruses (Tautz et al., 2015). Replication of the viral genome occurs in the cytoplasm. However, virion assembly and maturation take place in the endoplasmic reticulum (ER) and the Golgi apparatus (Blome et al., 2017).

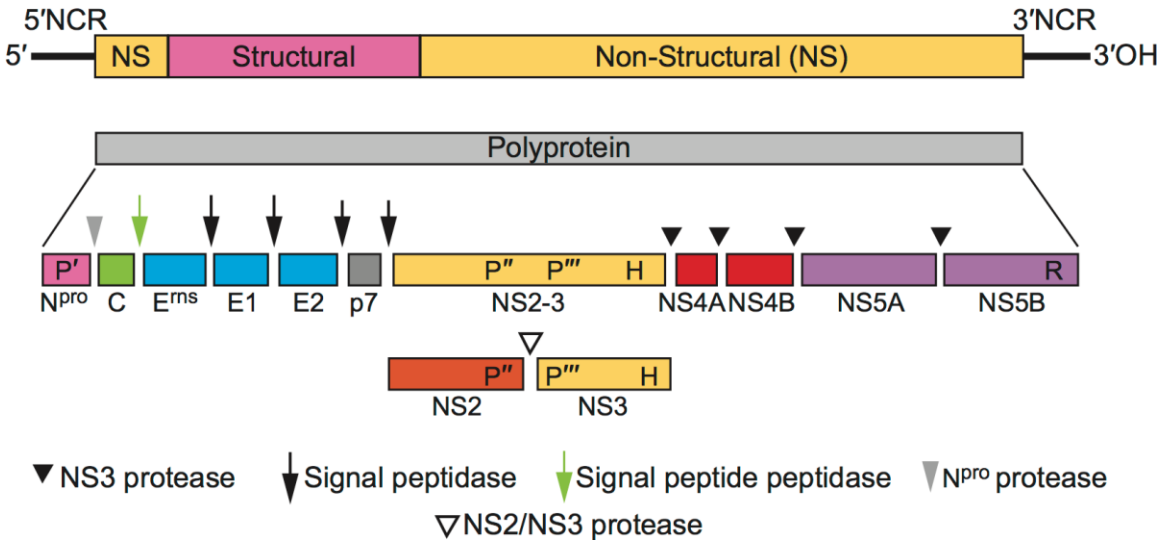

Figure 1.1. *Pestivirus* general genome structure color-coded based on belonging to structural or non-structural protein group (https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae/361/genus-pestivirus).

Structural Proteins

Within the structural proteins, there are three envelope proteins (E^{rns}, E1, and E2) that are involved in different stages of the virus life cycle (Lazar et al., 2003; Isken et al., 2013; Wang et al., 2014; Tautz et al., 2015; Wang et al., 2015; Riedel et al., 2017). These involvements are sorted into three categories based on their functions/interactions during the stages. The first category is based on their interactions with host cells. This includes attachment, entry, cell tropism, host specificity, receptor interaction, cellular protein interactions, fusion, endocytosis, and autophagy within the virus life cycle (Wang et al., 2015). The next category is rooted in how the *Pestivirus* envelope proteins interact with other viral proteins. Functions include dimerization through disulfide bonds and packaging/assembling virions (Wang et al., 2015). Lastly, the envelope glycoproteins are grouped based on functions during pathogenesis. The envelope proteins can interact with

cell receptors which determine both cell tropism along with pathogenicity. Other functions include inducing host humoral and cellular immunity while also evading the host immune system contributing to virulence (Wang et al., 2015)

Core Protein C

The core protein C is the first structural protein that is translated from the pestivirus genome. This highly basic protein is involved in virion packaging during its life cycle (Hause et al., 2015; Tautz et al., 2015; Pan et al., 2019). Its N-terminus end is produced by cleavage of the N^{pro}/C site by an autoprotease (N^{pro}) while the C/E^{rns} site is cleaved by a signal peptidase. While present in all pestivirus species, the length of C protein is variable. Experiments have demonstrated that even when a basic sequencing region was deleted, producing a smaller C protein, the virus was still able to replicate but at a lower efficiency. In recovered isolates lacking the C protein, NS3 (a serine protease described below) was able to compensate during virus assembly, just not at full efficiency (Tautz et al., 2015; Riedel et al., 2017).

E^{rns}

The first envelope glycoprotein encoded by the pestivirus genome is E^{rns}. While the other two envelope proteins (E1 and E2) contain a transmembrane domain, E^{rns} has a membrane anchor instead (Lazar et al., 2003; Lin et al., 2004; El Omari et al., 2013; Tautz et al., 2015; Wang et al., 2015; Pan et al., 2019). This glycoprotein is unique to only pestiviruses having an RNase function (Bruschke et al., 1997; Becher & Thiel, 1999; Lazar et al., 2003; Hause et al., 2015; Tautz et al., 2015; Postel et al., 2016; Riedel et al., 2017). It degrades single-stranded RNA. However, it does not interfere with viral genome replication as E^{rns} can only function in the host endoplasmic reticulum while replication

occurs in the cytoplasm (Tautz et al., 2015; Wang et al., 2015). No RNase activity is observed in the cytoplasm; thus, E^{rns} is not involved in replication of the viral genome. The E^{rns} protein is one of two pestivirus factors able to regulate the host interferon response to pestiviruses. When host cells uptake the virion, the RNase function of E^{rns} will degrade pestiviral RNA to levels that are not able to be recognized by the host immune system, thus no interferon response is mounted. The regulation of this response is what allows for noncytopathic pestiviral species to persist in animals (Bruschke et al., 1997; Lin et al., 2004; Hause et al., 2015; Tautz et al., 2015; Riedel et al., 2017; Pan et al., 2019). Isolates that did not contain a functional E^{rns} lead to less severe symptoms along with being protected against additional infections by pestiviruses. However, these isolated are characterized by lower transmission ability since there is less viral RNA being produced (Lin et al., 2004; Tautz et al., 2015). The RNase activity also has a specificity for NpU bonds (a 5' uracil base preceded by any other nucleotide. Activity is inhibited by Zn^{2+} and Mn^{2+} (Tautz et al., 2015).

E1

The E1 protein is the only pestivirus glycoprotein for which function and structure are not completely known. Antibodies for E1 are not produced in animals infected with pestiviruses. The size of E1 varies across pestivirus species, with the protein ranging from 25 to 33 kDa. Like the other pestiviral glycoproteins, several cysteine residues found in E1 are conserved across pestiviral species. The E1 protein is one of the two essential glycoproteins needed for the fusion of viral and cellular membranes during the viral lifecycle (Wang et al., 2014; Hause et al., 2015; Tautz et al., 2015; Pan et al., 2019). The heterodimer that E1 forms with E2 is important for pestivirus entry into host cells

(Tautz et al., 2015). Since E2 protein lacks the requirements to be considered the fusion peptide, it has been proposed that E1 may be the fusion peptide. However, there is still some speculation on this since E1 is just half the size of E2 and functionally, E1 is still not fully characterized (Wang et al., 2014; Tautz et al., 2015).

E2

The E2 protein is the last of the pestiviral envelope proteins encoded. It is the receptor-binding protein for pestiviruses and thus the target of neutralizing antibodies. It can be used in the diagnosis of pestivirus from infected animals. The E2 protein is also responsible for the species tropism found across the pestiviruses (Hause et al., 2015; Tautz et al., 2015; Zhang et al., 2018; Cagatay et al., 2019; Michelitsch et al., 2019). The C-terminus acts as the transmembrane anchor for the glycoprotein. The heterodimer formed between E2 and E1 is essential for pestivirus entry into host cells (Tautz et al., 2015).

Nonstructural proteins

N^{pro}

Unique to pestiviruses, N^{pro} is the first non-structural protein to be encoded by the pestivirus genome. This protein is an autoprotease that cleaves itself at the C-terminal end, creating the N-terminal end of the C protein (Hause et al., 2015; Tautz et al., 2015; Riedel et al., 2017; Pan et al., 2019). After cleavage, the N^{pro} protein becomes inactive. The cleavage site recognized by N^{pro} is highly conserved across different pestivirus species. However, while it is highly conserved, it is the only non-essential gene product in the pestivirus genome. Mutant viral isolates lacking a functional N^{pro} gene are still

viable, just with a reduced replication efficiency (Tautz et al., 2015). Additional studies focusing on the function of this non-essential protein revealed that it is most likely involved in blocking the host's type I interferon response triggered by the viral presence and thus protects the cell from apoptosis which propagates viral replication. It can block the type I interferon response by coordinating a zinc ion to interact with three cysteine residues (Tautz et al., 2015; Pan et al., 2019). Intracellular levels of interferon regulatory factor 3 are regulated by N^{pro} via activating its proteasomal degradation, also regulated by the binding of a zinc ion from the metalloprotein activity. The inhibition of the interferon response by N^{pro} and E^{rns} is what allows noncytopathic pestiviral species to lead to persistent infections (Tautz et al., 2015; Riedel et al., 2017).

p7

The p7 protein is classified as a non-structural protein because it is absent from virions. It is encoded in the genome downstream of the last structural protein, E2, and is released from the E2-p7 heterodimer by signal peptidase. While the E2-p7 heterodimer is not needed for viral entry or replication, p7 is required for viral propagation. It has been suggested that p7 acts as a viroporin, regulating ion transport involved in inducing proinflammatory cytokines (Hause et al., 2015; Tautz et al., 2015). Experiments connected p7 for being involved in the late stages of viral assembly by reducing the acidification of intracellular vesicles (Tautz et al., 2015).

NS2

The NS2 protein is a cysteine autoprotease that can cleave itself off from the NS2-3 protein (Hause et al., 2015; Tautz et al., 2015; Pan et al., 2019). After it separates from NS3, the C-terminus remains active which protects the NS2 protein from being cleaved

further by other proteases involved in the pestiviral infection. One main difference between NS2 in cytopathic and noncytopathic strains is the requirement of a cellular chaperone in noncytopathic strains which is needed to maintain the stability and function of the NS2 as a protease (Tautz et al., 2015).

NS2-3

While noncytopathic pestiviruses do not require uncleaved NS2-3 for viral replication, it does play an important role in virion morphogenesis. However, its exact function and role in this process are still not fully understood. In different pestivirus strains, when uncleaved NS2-3 was absent it led to a decrease in the production of infectious progeny even when the viral RNA replication process was intact. This is what constitutes the difference between cytopathic and noncytopathic strains and their ability to induce infection. Cytopathic pestiviral strains lack uncleaved NS2-3, which in turn leads to the inability to cause infection.

NS3

The NS3 protein possesses multiple functions dependent upon cleavage events and the part of the protein involved. In the N-terminal domain, there is a chymotrypsin-like serine protease encoded while the C-terminal contains both a helicase and NTPase domain (Isken et al., 2013; Hause et al., 2015; Tautz et al., 2015; Schwarz et al., 2017). For the N-terminal serine protease to function properly, NS4A acts as a cofactor to stabilize NS3. Once associated together, NS3 protease activity can splice the remainder of cleavage sites downstream of it (Isken et al., 2013; Tautz et al., 2015). If the protease activity of NS3 is affected, NS2-3/4A (NS4A is associated as a cofactor with uncleaved NS2-3) can compensate slightly for cleaving downstream sites but at a much slower rate.

The NS3/4A complex induces both caspase activation and apoptosis of host cells during pestiviral infection. Mutagenesis studies have shown second-site mutations in NS3 could compensate for the C protein's essential properties of viral replication when initial mutations in the C protein would result in either enlarged or lack of C protein. Based on this, phylogenetic studies have suggested that previously, NS3 had a viral packing function when the ancestral pestivirus lacked a capsid protein (Tautz et al., 2015).

NS4A

As mentioned above, NS4A acts as a cofactor for NS3 protease. The N-terminal contains a hydrophobic membrane while a cytosolic domain is in the C-terminal. The C-terminal could still provide protease cofactor activity if truncation occurred as long as it was by less than seven amino acids. The cells of animals infected with pestiviruses contain NS4A and an uncleaved precursor NS4A-B. The precursor does not have an exact role defined yet, but studies have demonstrated NS4A had an important part in virion morphogenesis. However, the exact reason and mechanism associated with this role was not been fully revealed (Tautz et al., 2015).

NS4B

The NS4B protein is hydrophobic and predicted to be part of the intracellular membrane. The function of NS4B is not fully characterized, but it is known to be an essential protein involved in pestiviral RNA replication (Tautz et al., 2015).

NS5A

The NS5A is involved in the regulation of viral RNA replication. The α-helix in the N-terminus functions as a membrane anchor holding NS5A to the intracellular membranes. The rest of the protein is split into three domains. The first domain is

important in viral replication since it contains a zinc-binding motif that coordinates a required zinc ion. This protein can tolerate some deletions and remain functional, allowing for various tags to be added to study different functions (Isken et al., 2013; Tautz et al., 2015). The N-terminal of the second domain of NS5A is not required for viral RNA replication or virion morphogenesis but the remainder of the protein is vital for these functions. Meanwhile, the third domain is not required for NS5A essential functions but is suggested to help aid in effective viral RNA replication (Isken et al., 2013). In CSFV, NS5A was able to regulate viral RNA replication through one or two ways. It could either bind to the 3'UTR of the viral genome with NS5A's RNA binding ability or it has direct protein-protein binding to NS5B regulating NS5B RNA-dependent RNA polymerase (RdRp) activity (Isken et al., 2013; Tautz et al., 2015). Three regions were identified as essential for the interaction of NS5A with NS5B. Interestingly, these regions are highly conserved across pestiviruses and even in HCV even though the remainder of the protein is less similar (Tautz et al., 2015).

NS5B

The sequence of the NS5B protein has characteristics of an RdRp and demonstrated to have polymerase activity *in vitro* (Isken et al., 2013; Tautz et al., 2015). However, for *in vivo* replication and proper propagation, NS5B also needs NS3. The NS5B protein contains folds characteristic of polymerases and RdRp with inhibitors interacting with both the finger and N-terminal domains of NS5B. This interaction/inhibition suggests these domains are essential for the enzymatic activity of NS5B. Experiments exchanging NS5B regions between different pestiviral species reveals a possible correlation between virulence and the activity of NS5B. Additional

research demonstrated that NS5B is also involved in virion morphogenesis (Tautz et al., 2015).

Atypical porcine pestivirus

Atypical porcine pestivirus (APPV) has been recently discovered all over the world and identified as the potential causative agent of congenital tremor type A-II (Postel et al., 2016; Zhang et al., 2016; Beer et al., 2017; Schwarz et al., 2017; Dessureault et al., 2018; Shen et al., 2018; Zhang et al., 2018; Kaufmann et al., 2019; Sutton et al., 2019; Guo et al., 2020). The congenital tremor can severely affect newborn piglets' ability to nurse, which can lead to possible malnutrition and death due to starvation (Zhang et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Zhang et al., 2018; Pan et al., 2019). Litters affected by congenital tremor experienced higher preweaning mortality, increased incidence of splayed legs, and smaller birth weights (Sutton et al., 2019). Within samples collected from these affected piglets, APPV was found to be the most predominant microbial species which provided evidence of APPV's involvement in congenital tremor type A-II (Sutton et al., 2019).

Phylogenetic studies comparing strains within and between countries across the world provide evidence of APPV being highly variable. This high variability currently limits the ability to identify all cases thus leading to an underestimate of the prevalence of APPV through the world (de Groof et al., 2016; Zhang et al., 2016; Beer et al., 2017; Postel et al., 2017; Dessureault et al., 2018; Shen et al., 2018; Kaufmann et al., 2019; Pan et al., 2019; Guo et al., 2020). Additionally, the 5'UTR appears to be highly conserved between isolated strains while there appears to be no strongly conserved regions within

the coding sequence (de Groof et al., 2016). Reports of APPV have continuously been identified in various countries for a few years. But, due to the high variability and lack of technology to detect APPV, cases from more than three decades ago have been tested and shown to contain the APPV genome. The retrospective studies thus show APPV has been in circulation longer than originally believed (de Groof et al., 2016; Schwarz et al., 2017; Kaufmann et al., 2019; Pan et al., 2019; Guo et al., 2020).

Quantification of viral RNA in piglets affected by congenital tremor have shown piglets less than a week old have the highest viral load compared to those later in life (de Groof et al., 2016; Pan et al., 2019). Sutton et al. (2019) quantified viral load in affected piglets, their unaffected littermates, and unaffected litters. The virus was detected by quantitative polymerase chain reaction (qPCR) assay in all affected piglets and their unaffected littermates but was absent from unaffected litters and the dams who were screened at the same time. The birth weights were significantly smaller in affected litters versus unaffected litters. However, affected piglets had higher birth weights than their unaffected littermates (Sutton et al., 2019). In terms of tissues, the highest viral loads were found within lymphoid organs. This finding provides evidence of APPV replication sites and the ability to suppress the host immune system, as seen in other pestiviruses (de Groof et al., 2016; Pan et al., 2019). Persistent infections of APPV are expected to occur with hosts being asymptomatic. These asymptomatic carriers have the potential to shed the virus and continue the transmission of APPV in herds. As with other pestiviruses, the persistent infections are most likely to be established due to the N^{pro} and E^{rns} proteins' role in immune system suppression (de Groof et al., 2016; Pan et al., 2019).

The details of pathogenesis, molecular biology, and transmission of APPV remain unknown (Postel et al., 2016; Pan et al., 2019). However, piglets affected with APPV and demonstrating congenital tremor often have a higher incidence of hypomyelination of white matter within the spinal cord and/or brain stem. Myelin sheaths have also been observed to be smaller than unaffected piglets (Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Mósena et al., 2018; Pan et al., 2019). There also appears to be no significant correlation between detected viral load and the severity of piglet symptoms. As with other details, the exact reason or mechanism of why there is no correlation remains unknown (Arruda et al., 2016; de Groof et al., 2016; Dessureault et al., 2018). Piglets affected with congenital tremor due to APPV who survived to weaning despite their symptoms were able to grow out of it with the tremors subsiding later in life (de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Pan et al., 2019; Guo et al., 2020).

Inoculation studies have demonstrated APPV infects piglets during gestation. However, as with general pestiviruses, infection of the fetuses must occur before the immune system develops for the fetus (Arruda et al., 2016; Postel et al., 2016; Dessureault et al., 2018; Kaufmann et al., 2019). Interestingly, multiple groups have found litters from gilts were more likely to be infected by APPV and have congenital tremor versus later parities of dams. This trend of infected litters based on the reproductive state of the mother suggests some type of immunity can develop and protect later offspring (de Groof et al., 2016; Postel et al., 2016; Dessureault et al., 2018; Mósena et al., 2018). On the other hand, the route of transmission is still unknown. It has been hypothesized the virus can infect from shedding in semen from boars. While the boars

were not affected with tremors at the time of breeding, they may have experienced congenital tremors as piglets where a persistent infection could have been established (Schwarz et al., 2017; Dessureault et al., 2018; Kaufmann et al., 2019; Pan et al., 2019; Guo et al., 2020). However, the exact mechanisms of such possible persistent infections due to APPV are unknown.

Attempts to isolate and culture different strains of APPV have been conducted with limited success. The key to this success is obtaining samples from affected piglets before they receive colostrum and thus maternal antibodies and using this as the inoculate (Schwarz et al., 2017). On the other hand, some strains have been unsuccessful in culturing. Pan et al. (2019) hypothesized this difficulty might be attributed to the high variability between strains with some strains not being robust enough to culture.

Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus (BVDV) was first described in 1989 with infected animals displaying signs of hemorrhagic disease leading to a high mortality rate. The most infected tissues were associated with the immune system, specifically lymphoid tissues. Infection can begin *in utero* as BVDV can cross the placenta. BVDV also can cause mucosal disease (MD). Cattle younger than two years of age who develop MD this way have bloody diarrhea, anorexia, fever, and overall weakness resulting in 100% mortality within two weeks of first presenting symptoms (Tautz et al., 2015).

As with general pestivirus, BVDV is a single-stranded, positive-sense RNA virus. Its genome is approximately 12.3 kilobases with only one open reading frame encoding 13 proteins like in most of the Pestiviruses (Isken et al., 2013; Deng et al., 2020).

Phylogenetic analysis differentiated two genetic species of BVDV (BVDV-1 and BVDV-2) based on N^{pro} and E2 proteins. The BVDV-1 strain is seen around the world while BVDV-2 is primarily seen in North and South America (Deng et al., 2020).

Border Disease Virus

Border disease virus (BDV) is a pestivirus mainly affecting sheep. Many symptoms and characteristics that are found in ruminants affected by BVDV are like those found in sheep infected with BDV. Similarly, BDV can cross the placenta which infects fetuses. *In utero* infections can lead to malformation of the fetus, fetal death, and/or persistent infections. Lambs who experience persistent infections have continuous viral replication but do not always display symptoms. Animals who are persistently infected and show symptoms such as ataxia, lower birth weights, and tremors referred to 'hairy shaker syndrome' (Tautz et al., 2015; Braun et al., 2019). The 'hairy shaker syndrome' is believed to be caused by neural lesions in fetuses, either dysmyelination or hypomyelinogenesis. However, most of the lambs affected by this syndrome, tend to grow out of it and survive (Schwarz et al., 2017).

This virus is a positive-sense, single-stranded RNA virus. Its genome encodes a single open reading frame that is processed into 13 proteins. For phylogenetic purposes, BDV is grouped using the highly conserved 5'UTR and N^{pro}. Based on this, eight subtypes have been assigned, designated BDV-1 through BDV-8. While BDV is prevalent throughout the world, the individual subtypes are associated with specific countries (Braun et al., 2019).

Classical Swine Fever Virus

Classical swine fever virus (CSFV) leads to extremely severe symptoms, acute hemorrhagic disease, and a high mortality rate. However, the most recent strains are less virulent and symptoms less severe compared to initial cases. While current mortality rates are not as high as they were, there are now increased cases of chronic infections. Typically, CSFV can cause infected animals to have pyrexia, respiratory symptoms, gastrointestinal issues, and hemorrhages of skin and inner organs. Sequence comparisons between avirulent and virulent strains have demonstrated there is not a strict region difference which could account for the variation in severity of the disease. It is suspected that multiple regions in the CSFV genome can contribute to the virulence of the strain (Tautz et al., 2015).

Like other pestiviruses, CSFV is a single-stranded, positive-sense RNA virus. Its genome is approximately 12.3 kilobases in size. There is only one open reading frame that encodes a polyprotein which is co- and post-translationally processed to produce 13 mature proteins, characteristic of pestiviruses. Viral replication occurs in the cytoplasm while virion assembly and maturation occur in the ER and Golgi apparatus. Phylogenetic analysis utilizing E2, N^{pro}, C, E1, and E^{rns} differentiated CSFV into three major genotypes with further subdivisions. Areas including South and Central America, Eastern Europe, Asia, and India are some of the places where CSFV is causing endemics (Blome et al., 2017).

CONGENITAL TREMOR IN PIGLETS

General Background

The phenomenon of shaking in piglets has been observed for over 100 years (Arruda et al., 2016; de Groof et al., 2016; Zhang et al., 2018). The “dancing pigs”, as it was originally described, is characterized by bilateral skeletal contractions that will begin at birth or soon after and will typically halt while the piglet is resting. The tremors can impede a piglet’s ability to nurse and thus can lead to malnutrition and possibly death due to malnutrition (Done et al., 2012; Arruda et al., 2016; de Groof et al., 2016; Zhang et al., 2017; Schwarz et al., 2017; Dessureault et al., 2018; Shen et al., 2018; Zhang et al., 2018; Kaufmann et al., 2019; Michelitsch et al., 2019; Pan et al., 2019).

Observations of piglets affected by congenital tremor have demonstrated the association with demyelination of the brain and spinal cord (Blomström et al., 2014). Congenital tremor is differentiated into either type A or type B based on the presence or absence of morphological lesions within either the brain and/or spinal cord, respectively. The different subtypes of congenital tremor have been associated with several factors including infection with certain viral strains, pig genetic backgrounds, and intake of some toxic chemicals (Arruda et al., 2016; de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Shen et al., 2018; Zhang et al., 2018; Kaufmann et al., 2019; Michelitsch et al., 2019; Pan et al., 2019).

Type A

Type A-I

Type A-I is caused by classical swine fever virus (CSFV) in fetuses during gestation and typically has a high mortality rate (Blomström et al., 2014; Arruda et al.,

2016; de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Shen et al., 2018; Kaufmann et al., 2019; Pan et al., 2019).

Type A-II

Type A-II congenital tremor has previously not had a causative agent. While mortality due to the tremor itself is low, most of the deaths are associated with malnutrition due to difficulties in nursing (Done et al., 2012; Blomström et al., 2014; Arruda et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Zhang et al., 2018; Kaufmann et al., 2019; Pan et al., 2019). With the advances in next-generation sequencing, various research groups have identified atypical porcine pestivirus as a potential source/cause of type A-II congenital tremor (Arruda et al., 2016; de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Mósena et al., 2018; Kaufmann et al., 2019; Michelitsch et al., 2019; Pan et al., 2019).

Type A-III

This type of congenital tremor is one of two types that is considered hereditary in a sex-linked recessive pattern of inheritance. Type A-III results from hereditary lesions found in male Landrace pigs (Done et al., 2012; Arruda et al., 2016; de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Pan et al., 2019). Similar to other mammals and their related tremors, the proteolipid protein was found to be the most likely cause of type A-III congenital tremors found in the Landrace pigs. This protein is highly conserved and inherited in an X-linked fashion (Baumgartner & Brenig, 1996).

Type A-IV

Type A-IV is the other type of congenital tremor which is inherited and only seen in Saddleback pigs. This is inherited in an autosomal recessive pattern (Done et al., 2012; Arruda et al., 2016; de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Pan et al., 2019).

Type A-V

Type A-V congenital tremors are brought about through intoxication or intake of certain chemicals during gestation. Chemicals such as metrifonate and trichlorfon have been linked to producing these types of tremors in swine (Done et al., 2012; Arruda et al., 2016; de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Shen et al., 2018; Pan et al., 2019).

Type B

While type A congenital tremor is firstly identified by the presence of morphological lesions in either the brain or spinal cord, type B is characterized by the absence of these (Done et al., 2012; Blomström et al., 2014; Arruda et al., 2016; de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018).

HOST GENETICS ROLE IN SWINE VIRAL DISEASES

Porcine Circovirus Type 2

Porcine circovirus type 2 (PCV2) is a single-stranded DNA virus and is the smallest DNA virus that can infect mammals. This virus is the main cause of porcine circovirus associated diseases (PCVAD) and has a negative economic impact on the swine industry. However, all pigs infected with PCV2 do not necessarily progress to

PCVAD (Meng, 2013; Goedbloed et al., 2014; Walker et al., 2018). Recent studies have identified a host genetic component that can explain the variation in severity and number of cases (McKnite et al., 2014; Walker et al., 2018). Walker et al. (2018) identified two genomic regions associated with viral replication of PCV2 and immune response phenotypes, one near the swine leukocyte antigen complex class II (SLAII) on chromosome 7 (*Sus scrofa* chromosome 7, SSC7) and one at the proximal end of SSC12. Within the SLAII region (SSC7), two single nucleotide polymorphisms (SNPs) identified explained 3.8% of the genetic variance in PCV2 viral load. Meanwhile, the major SNP within the proximal end of SSC12 explained 9.3% of the genetic variance of viral load and 6.2% of the phenotypic variance. *In vitro* validation analysis, using siRNA and CRISPR/Cas9 gene editing, validated the direct role of *SYNGR2* (SSC12), a positional candidate gene in this region, in PCV2 replication. A missense mutation (*p.Arg63Cys*) in a conserved protein domain of *SYNGR2* explained most of the observed variation in PCV2 viral load. The SNP variant was conserved across mammalian species with the cysteine variant only being observed in swine and associated with lower viral load.

Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the major swine viral pathogens causing major loss in revenue in the swine industry. It is a single-stranded RNA virus that can induce decreased fertility and increased respiratory issues (Boddicker et al., 2012; Serão et al., 2014; Koltes et al., 2015; Harding et al., 2016; Dekkers et al., 2017; Walker et al., 2019). Vaccine development has not been able to efficiently prevent infections because the virus displays high levels of antigenic and

genetic drift in both structural and nonstructural proteins. It is also able to evade early immune response, adding to the severity of the consequences. (Boddicker et al., 2014; Serão et al., 2014; Koltes et al, 2015; Dekkers et al., 2017). Recent studies have suggested that host genetics could play a role in PRRSV susceptibility (Boddicker et al., 2014; Serão et al., 2014; Koltes et al., 2015; Dekkers et al., 2017; Walker et al., 2019). Having a genetic component identified would allow breeders to select for increasing natural resistance in the domestic population of pigs while reducing the income loss and animal welfare.

Boddicker et al. (2014) found evidence of genomic regions located on SSC4 associated with PRRSV viral load and weight gain at 42 days post experimental infection. The region was in high linkage disequilibrium and included genes from the guanylate-binding protein family, genes activated by interferons, which are released as part of the immune response to infection. In the following work, Koltes et al. (2015) found a possible causative mutation in guanylate binding protein 5 gene (*GBP5*) which led to an alternative splice site, resulting in the variation in response to PRRSV by the host. This gene has previously been shown to be involved in mediating inflammasome assembly during an immune response.

Walker et al. (2019) conducted genome-wide association analyses of two genetic lines of pigs experimentally infected with PRRSV. Two major genome regions were identified which accounted for approximately 1.2% of the genetic variation of PRRSV-specific antibodies in serum or lung. One region mapped to chromosome 7 near the SLAII complex (which has also been associated with susceptibility to other swine pathogens). The window identified in Walker et al. (2019) was located within the

previous 7 Mb window identified by Serão et al. (2014; for PRRSV-specific antibodies) but had a different top SNP. The other major genome region was novel, mapped to SSC17 with the major SNP covering two genes (*LONRF1* and *PRAG1*).

Swine Leukocyte Antigen Complex II

The swine leukocyte antigen (SLA) complex is located near the centromeric region of SSC7. This region is one of the most gene-dense regions within the swine genome despite it being the smallest major histocompatibility complex (MHC) among mammals (Gustafsson et al., 1990; Kanai et al., 1999; Chardon et al., 2000; Smith et al., 2005a; Smith et al., 2005b; Renard et al., 2006; Ho et al., 2009; Luetkemeier et al., 2009; Lunney et al., 2009; Ho et al., 2010; Park et al., 2010; Minh Thong et al., 2011; Hess et al., 2018). The SLA complex is composed of three gene clusters/regions designated class I, class II, and class III. The genes in the complex are a major determinant in the immune response to disease and vaccine response (Gustafsson et al., 1990; Kanai et al., 1999; Chardon et al., 2000; Lee et al., 2005; Smith et al., 2005a; Renard et al., 2006; Ho et al., 2009; Lunney et al., 2009; Ho et al., 2010; Park et al., 2010; Minh Thong et al., 2011; Shinkai et al., 2012; Serão et al., 2014). Of interest is SLAII which is highly homologous to the human MHCII. Genes in this region are involved in pathways for antigen presentation upon infection. The SLA-DR and SLA-DQ are protein binding antigens expressed on the surface of antigen-presenting cells like macrophages, B cells, and dendritic cells. While they are preferentially expressed on CD8+ T cells (with SLA-DR more so than SLA-DQ), they are also expressed at low levels on circulating cells. However, the main role of SLAII antigens is to present the exogenous peptides to CD4+

helper T cells which can elicit an immune response (Gustafsson et al., 1990; Chardon et al., 2000; Lee et al., 2005; Smith et al., 2005a; Ho et al., 2009; Luetkemeier et al., 2009; Lunney et al., 2009; Ho et al., 2010; Minh Thong, et al., 2011; Feng et al., 2012; Shinkai et al., 2012; Hess et al., 2018; Walker et al., 2019).

Within the SLAII complex, the SLA-DRB and SLA-DQB genes encode β chains. Within this locus, there is a functional *SLA-DRB1* gene and four pseudogenes (*SLA-DRB2-5*). There is also one functional *SLA-DQB1* gene and a pseudogene (*SLA-DQB2*). A high degree of polymorphisms can be found within these functional genes of SLAII. (Baba et al., 1999; Kanai et al., 1999; Chardon et al., 2000; Renard et al., 2006; Lunney et al., 2009; Ho et al., 2009; Luetkemeier et al., 2009; Ho et al., 2010; Minh Thong et al., 2011). Due to the high degree of polymorphism and the size of these genes, until the development of long-read RNA-seq (Oxford Nanopore and Pacific Biosciences), it has been difficult to sequence this gene in a single continuous amplicon. Most polymorphisms identified are located within the antigen-binding region, extending the ability to recognize various pathogens to signal the immune system to respond.

Genome-wide association studies have implicated the SLAII region in association with various viral pathogens. As previously mentioned, Serão et al. (2014) and Walker et al. (2019) found a major QTL near the SLAII region that partially explained the variation observed in the PRRSV-specific antibody levels in infected pigs. Also, a QTL mapped to the SLAII region was also reported for the viral load of another important swine virus, PCV2 (Walker et al., 2018).

Similar studies have also associated the human MHC region to diseases as well, which is not surprising since there is a high degree homology between regions of SLA

and MHC. Studies have associated specific changes in residues in the binding groove of different MHC proteins with autoimmune diseases. A classic example is studies observing the *HLA-DQB* amino acid position 57 and its relationships with type I diabetes. Individuals with specific *HLA-DQB* haplotypes were shown to be almost completely resistant to type I diabetes (Jones et al., 2006; Trowsdale & Knight, 2013). The *HLA-DQB* gene has also been implicated as a primary source of narcolepsy (Jones et al., 2006; Trowsdale & Knight, 2013). Multiple sclerosis is an autoimmune disease with a multifactorial determinism that also includes *HLA-DRB1* variants (Trowsdale & Knight, 2013).

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CHAPTER 2: DETECTION OF ATYPICAL PORCINE PESTIVIRUS GENOME IN NEWBORN PIGLETS AFFECTED BY CONGENITAL TREMOR AND HIGH PREWEANING MORTALITY*

[1]Parts of this chapter were published in Journal of Animal Science (2019)

INTRODUCTION

Recently, there has been an increase in the number of cases of growing pigs exhibiting a range of neurological conditions such as muscle tremors that can progress to paralysis and death (Swine Health Information Center; https://www.swinehealth.org). A number of viral sources leading to these conditions have been discovered, including atypical porcine pestivirus (APPV), porcine teschovirus, and porcine sapelovirus. Using metagenomic sequencing of swine clinical samples, Hause et al. (2015) were the first to identify and characterize APPV as a new species of Pestivirus. At that time, no symptoms were associated with the presence of this newly characterized virus but later studies were able to associate APPV with the presence of congenital tremor in newborn piglets and with varying degrees of hypomyelination in both brain and spinal cord (Arruda et al., 2016; Schwarz et al., 2017; Mósena et al., 2018). APPV has been detected around the world and characterized by important genetic variations between strains and geographical regions (Hause et al., 2015; Postel et al., 2016; Beer et al., 2017; Zhang et al., 2017;

[1] Sutton, K. M., Lahmers, K. K., Harris, S. P., Wijesena, H. R., Mote, B. E., Kachman, S. D., Borza, T., & Ciobanu, D. C. (2019). Detection of atypical porcine pestivirus genome in newborn piglets affected by congenital tremor and high preweaning mortality. *Journal of Animal Science, 97*(10), 4093-4100. https://doi.org/10.1093/jas/skz267.

Mósena et al., 2018). This substantial genetic variation could lead to difficulties in detecting the presence of certain strains using traditional diagnostic tests such as quantitative PCR (qPCR). In the recent years, metagenomic analysis was used successfully in identifying novel pathogens and capturing genetic diversity among microbial genomes without any prior requirement of genomic information. In this study, we employed metagenomic sequencing to discover and characterize a novel strain of APPV associated with piglets exhibiting congenital tremor and high preweaning mortality.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use of Committee of the University of Nebraska-Lincoln.

Sample and Data Collection

A research farm characterized by high-health status, a comprehensive vaccination program, and naïve for other important pathogens started to observe first cases of congenital tremor in August of 2017. Blood samples were collected from newborn piglets from normal litters and from litters affected by congenital tremor and housed in the same facility. The samples were centrifuged at 2,350 × g at 4 °C for 30 min and the serum was stored at −80 °C. The litters were generated by maternal crossbred dams inseminated with commercial Duroc semen. The source farm has vaccination programs for porcine circovirus 2, porcine parvovirus, *Erysipelothrix rhusiopathiae*, *Clostridium perfringens type C*, leptospirosis, and colibacillosis. The farm is naïve for other important viral

pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV) or porcine epidemic diarrhea virus. Following initial cases of congenital tremor, a controlled exposure data set was generated (March 2018) by subjecting maternal crossbred gilts (n = 91) to an inoculate obtained from fetal fluids and membranes collected from sows that produced litters affected by congenital tremor. Litter size traits, including total number of piglets born (TNB), number of mummified and stillborn, and presence of splayed legs were recorded at farrowing. The weight of the piglets was measured at birth and at weaning.

Pathological Examination

Cross-sections of the brain at the level of the cerebellum were fixed by immersion in 10% neutral buffered formalin and then routinely processed overnight on a Leica Peloris II tissue processor. The sections of the processed brain were embedded in paraffin blocks and cut to 4 µm thick using a Leica RM2255 microtome, stained with hematoxylin and eosin on a Leica ST5020 H&E stainer, and cover-slipped with a Leica CV5030 coverslip. A veterinary anatomic pathologist who is board certified by the American College of Veterinary Pathologists performed all histological evaluations.

Detection of the Potential Viral Source of Congenital Tremor Using Oxford Nanopore Sequencing

Initial analysis of the potential source of congenital tremor was based on nucleic acids isolated from serum of affected piglets using QIAamp Circulating Nucleic Acid kit (Qiagen). To generate ~1 µg of complementary DNA (cDNA) required for library

preparation and Oxford Nanopore sequencing, a preamplification approach was implemented using nanomer-extended primers and experimental conditions as described by Greninger et al. (2015). This approach was tested successfully for sequencing various clinical samples for the detection of bacteria, fungi, parasites, DNA, and RNA viruses (reviewed in Greninger et al., 2015). Briefly, the first strand of cDNA was obtained from pooled RNA of 4 affected samples using Superscript III reverse transcriptase (Life Technologies) and random nanomers-extended primers (5′-GTTTCCCACTGGAGGATA-N9 -3′). The second strand of cDNA was synthesized using Sequenase DNA polymerase (Life Technologies). The cDNA obtained was subjected to preamplification using a primer based on the extended sequence of the random nanomers (5′-GTTTCCCACTGGAGGATA-3′). Metagenomic cDNA library was generated by Oxford Nanopore Ligation Sequencing kit 1D (SQK-LSK108, Oxford Nanopore Technologies), including the following steps: 1) end-repair with NEBNext Ultra II End Repair/dA-Tailing Module (New England BioLabs), 2) AMPure XP beads (Beckman Coulter) based purification, 3) adapter ligation with NEB Blunt/TA Master Mix, 4) AMPure XP beads-based purification, and 5) elution in 15-µl buffer (Oxford Nanopore Technologies). The obtained libraries were sequenced on an Oxford Nanopore MinION using R9.4 flow cells loaded with 75 µl of sequencing mix (12-µl cDNA library, 35-µl buffer with fuel mix, 25.5-µl library loading beads, and 2.5-µl ddH20) and using Nanopore MinKNOW 1.15.6 version as described in the manufacturer's protocol.

Genome Sequencing and Assembly of the Novel APPV Strain

Sequencing of the novel APPV strain was based on full-genome preamplification using Primal Scheme (http://primal.zibraproject.org), a web-based tool for primer design (Quick et al., 2017). The primers were designed based on the initial Nanopore sequencing of the MK728876 strain and its genetically closest complete genome sequence of a porcine atypical pestivirus 1 (MF167291.1). The amplicon length was set up to 400 bp with an overlap of 50 bp leading to 39 primer-pair sets. Genome preamplification of the MK728876 strain was based on 22 pooled cDNA samples characterized by high viral titer. First-strand cDNA synthesis of the viral RNA was carried out using Superscript III reverse transcriptase followed by PCR (GoTaq Flexi DNA polymerase; Promega) and agarose gel electrophoresis. Equal amounts of amplicons from each primer set were pooled (390 µl) followed by purification using AMPure XP beads (1× ratio of cDNA to beads). DNA concentration was evaluated using Qubit 1.0 fluorometer (Life Technologies). Sequencing of the amplicons was performed using Ligation Sequencing kit 1D (SQK-LSK108) as describe above. FAST5 reads that contained raw Nanopore signals were base-called using Albacore 2.3.3 (https://nanoporetech.com/). Adapters were removed using Porechop Nanopore application v0.2.3.

Metagenomic classification of the initial Nanopore sequencing data was performed using Centrifuge 1.0.3. package (https://ccb.jhu.edu/software/centrifuge/manual.shtml centrifuge-class option) (Kim et al., 2016). The “centrifuge-build” indexer was used to generate an index on all complete, bacteria, viral, archaea, and host (swine) genomes while the “centrifuge-class” option was used to classify the sequencing reads to specific genomes. Pavian application

(https://ccb.jhu.edu/software/pavian/) was used in the analysis of the metagenomic classification results (Breitwieser & Salzberg, 2016).

Genome assembly of the new APPV strain was based on several tools initially developed for sequencing the Zika virus (https://github.com/zibraproject/zika-pipeline). Briefly, FAST5 reads that contained raw Nanopore signals were base-called using Albacore 2.3.3 (https://nanoporetech.com/). Adapters were removed using Porechop Nanopore application v0.2.3. The reads were mapped to the reference genome (MF167291.1) using minimap2 (https://github.com/lh3/minimap2) and converted to BAM format by "samtools view." The script align_trim.py tool was used for trimming the preamplification primers and for normalization of the coverage. The sequencing variants were called using "nanopolish variants" (https://github.com/jts/nanopolish) application, while consensus sequence was generated by script margin_cons.py.

qPCR Testing of APPV

Estimates of the number of APPV copies or viremia in serum were performed using cDNA based on viral RNA isolated by MagMAX Viral RNA Isolation kit (Applied Biosystems) and reverse transcribed by Superscript III reverse transcriptase (Invitrogen). Viral RNA was extracted from blood collected at an average age of 6.6 ± 0.3 d from all available piglets from 25 litters (out of 91 total) affected by congenital tremor (n = 171). The APPV presence was determined based on a qPCR specific to the novel APPV MK728876 strain (Forward: 5'-CGA CTG AAG GCA GGA AAT TTG-3'; Reverse: 5'-GCT ACA AAC ACC AGG CAA TTC-3'; Probe: 5'-TC TCT ATA G/Zen/T GGC AAC CCC AAC CTC T-3') using TaqMan Master Mix (Life technologies) and CFX384 Real-

Time PCR (BioRad). The qPCR assay was initially tested on viral cDNA samples collected from piglets exhibiting congenital tremor (n = 82), their unaffected littermates (n = 2), and their dams (n = 17) but also from piglets from unaffected litters (n = 25). The number of APPV copies/mL of serum (log10) in experimental samples were obtained based on a standard dilution curve of a positive control DNA of known titer.

Statistical Analyses

The relationship between the presence or absence of congenital tremor and occurrence of stillborn, mummies, and splayed legs across litters was evaluated by Pearson chi-square contingency test, coding the presence or absence of the targeted phenotypes in each litter. A linear mixed model was used to investigate the association between the presence/absence of congenital tremor and birth weight, weaning weight, and average daily gain (ADG) during lactation. The model included litter as a random effect, litter size (TNB) as covariate, sex, and occurrence of congenital tremor as fixed effects. A linear mixed model was used to investigate the relationship between APPV viral titer and presence/absence of congenital tremor; the model included birthweight as a covariate, presence/absence of tremor as fixed, and litter as random effects.

Phylogenetic Analyses

To assess the evolutionary relationship of the novel strain with the other APPV strains, the DNA sequence and the viral polyprotein sequence were compared with that of 30 other complete APPV genome and protein sequences, respectively. Genomic data were retrieved from GenBank. DNA and protein alignments were performed using

MUSCLE (Edgar, 2004) implemented in MEGA X (Kumar et al., 2018). The phylogenetic tree based on DNA alignment was inferred by using the maximum likelihood method and Tamura–Nei model (Tamura & Nei, 1993) while that based on the protein alignments by using the maximum likelihood method and JTT matrix-based model (Jones et al., 1992).

RESULTS AND DISCUSSIONS

Litters Affected by Congenital Tremor Had A Higher Incidence of Preweaning Mortality

A swine research farm characterized by high-health status and a comprehensive vaccination program started to observe the first cases of congenital tremor in August of 2017. The affected litters were generated by maternal crossbred dams inseminated with commercial Duroc semen. Potential causes of congenital tremor were investigated histologically and by commercial and literature-based qPCR (Arruda et al., 2016) using multiple tissues. Postmortem histologic examination of affected piglets showed small numbers of scattered white matter vacuoles in the cross-sections of the cerebellum and underlying brainstem collected around the level of the pons to myelencephalon. The vacuoles did not have a single distinct region affected but appeared more commonly along the white matter of the cerebellar folia as well as near the junction of the cerebellum and brainstem. The vacuoles varied in size with most ranging from 10 to 30 μm in diameter. Similar findings were reported by other studies in piglets affected by congenital tremor (Schwarz et al., 2017; Mósena et al., 2018). For example, Schwarz et al., (2017) found vacuoles in the white matter of the cerebellum in the affected piglets,

while rare or absent vacuoles were found in the unaffected littermates and no vacuoles were found in the healthy control piglets. Commercial diagnostic qPCR testing for RNA viruses, including APPV, porcine teschovirus, and porcine sapelovirus, known to induce neurological symptoms in pig were all negative in the affected piglets.

A batch of 91 gilts of similar genetics and born on the same farm were subjected to an antigen oral exposure approximately 54 days prior to breeding using fetal fluid from the first group of litters displaying congenital tremor. In the exposed sows, 45.0% of the litters and 30.8% of all piglets were affected by congenital tremor (Table 2.1). The frequency of piglets with congenital tremor in the affected litters varied from 5.9% to 100%. The time of farrowing (September 4, 2018, to September 27, 2018) did not appear to influence the occurrence of congenital tremor across litters. Overall, the preweaning mortality in piglets affected by congenital tremor was 46.4% compared to 15.3% in unaffected piglets (in both affected and unaffected litters). The preweaning mortality of the unaffected littermates from affected litters was 24.2% compared to 12.7% of the piglets from unaffected litters. Also, litters affected by congenital tremor had a higher incidence of the average preweaning mortality (17.2%) compared to unaffected litters (6.5%). About half (53.7%) of the mortality in the affected piglets occurred on the first day of life, while three-fourths of the mortality occurred in the first 5 d of life (76%). An impact of congenital tremor on preweaning mortality was previously reported by Schwarz et al. (2017) in 2 farms from Austria in 2015 (mortality up to 25%) and 2016 (up to 30%).

Trait/CT Phenotype	Affected Litters		Unaffected Litters
	CT	No CT	
Number of Litters	41		50
Number of piglets born alive	373	186	652
Incidence of stillborn, %	8.92		8.0
Incidence of mummies, %	3.60		3.79
Preweaning mortality, %	46.4	15.3	12.7
Incidence of splayed legs, %	33.0	2.15	0.46
Birth weight (means +/- SE, kg)	1.22 ± 0.02	1.09 ± 0.03	1.3 ± 0.02
Log_{10} copies of APPV/mL of serum (n=171)	6.55 ± 0.21	6.25 ± 0.21	-

Table *2*.1. Summary of phenotypic information of a batch of litters affected by congenital tremor (CT) generated by sows (n=91) exposed to an APPV-based antigen ~54 days prior to breeding.

Litters Affected by Congenital Tremor Had A Higher Incidence of Splayed Legs and Smaller Birth Weights

A significant relationship was observed between the frequency of congenital tremor and splayed legs (Pearson $\chi 2 = 40.2$, $P < 0.0001$). Incidence of splayed legs was observed in the majority of the litters affected by congenital tremor (73.2%), while, in the unaffected litters, the incidence was limited (6.0%). The presence of splayed legs in piglets affected by tremor (33.0 %) was larger compared to unaffected piglets (0.8 %) (Table 2.1). The average frequency of splayed legs in the litters affected by congenital tremor was 22.4%, while, in the unaffected litters, the average frequency was marginal

(0.5%). This relationship between congenital tremor and incidence of splayed leg was reported in other studies (Arruda et al., 2016; de Groof et al., 2016). The presence of piglets with congenital tremor across litters was not associated with the presence of stillborn ($P > 0.98$) or mummies ($P > 0.26$).

A significant relationship between the occurrence of congenital tremor and birth weight was observed across and within litters ($P < 0.0001$). Piglets from litters not affected by tremor had greater birth weight (1.30 ± 0.02 kg) compared to piglets exhibiting tremor (1.22 ± 0.02 kg) and their unaffected littermates (1.09 ± 0.03 kg) from affected litters ($P < 0.05$). Interestingly, piglets exhibiting congenital tremor had larger birth weights compared to their normal littermates ($P < 0.0001$). A similar finding was reported by Harding et al. (2017) using the pregnant gilt model to study host response in PRRSV infection. They hypothesized that large fetuses appear to be more susceptible to PRRSV due to larger placentae or higher nutrient requirements. The presence of congenital tremor across and within litters had no effect on weaning weight or on ADG during lactation ($P > 0.12$).

APPV, The Predominant Microbial Species In The Serum of Pigs Affected By Congenital Tremor

Oxford Nanopore sequencing was employed for identification of the potential viral source associated with congenital tremor by metagenomic sequencing of cDNA libraries generated from serum samples of piglets affected by congenital tremor. The initial Nanopore sequencing data included 66,898 sequencing reads with an average read length of 568 bases. The sequencing data was classified to 228 reference genomes using

the Centrifuge 1.0.3 package. APPV (NC_030653.1), with 205 mappable reads obtained, was the most abundant microbial species observed in the samples of the affected pigs (Figure 2.1). In addition, other sequencing data classified as part of the virome included 34 reads uniquely mapped to porcine circovirus 3 (PCV3, NC_031753.1). The presence of PCV3 was acknowledged in other swine metagenomic studies (Palinski et al., 2017). The 4 reads originally classified as *Shamonda orthobunyavirus* (NC_018464.1) were also mapped to host *Sus scrofa* genome. These sequences are most likely part of the host genome since their average alignment coverage was 74% to *S. scrofa* genome compared to only 7% to *S. orthobunyavirus*.

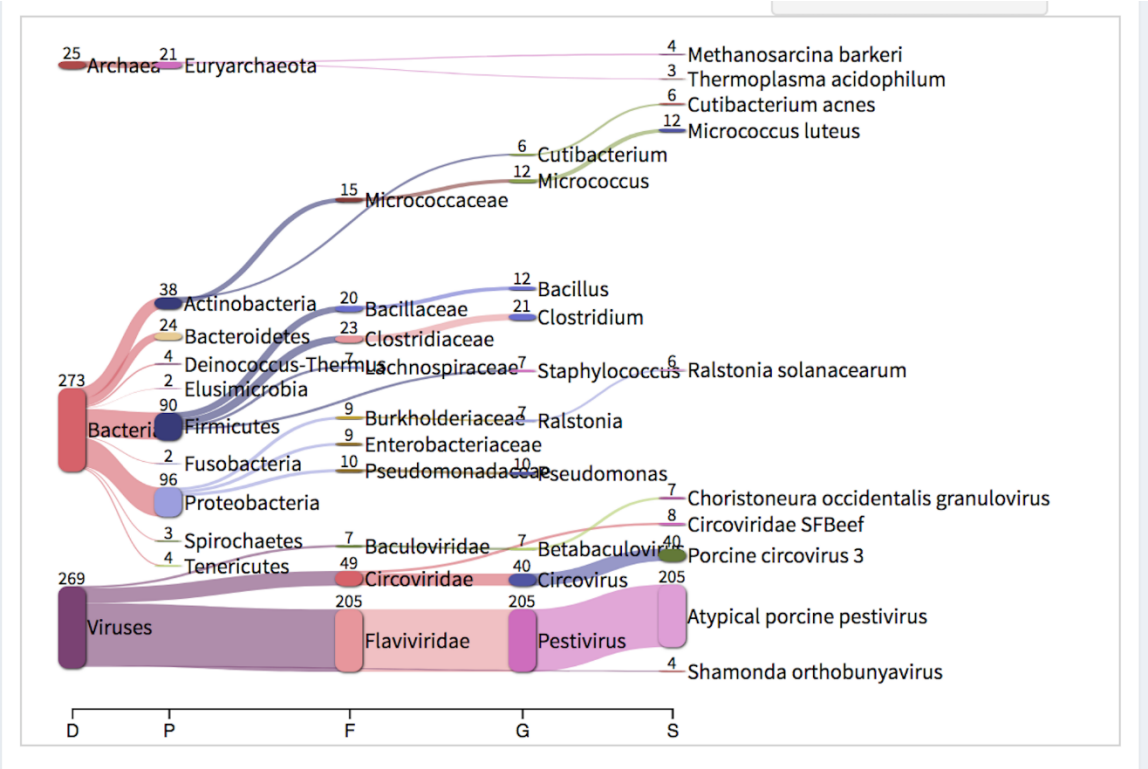

Figure 2.1. Alignment of Oxford Nanopore sequencing reads to the reference genomes based on tissues collected from piglets affected by congenital tremor indicated that the most abundant microbial species was APPV.

While metagenomic analysis was used in the discovery efforts of the potential sources of congenital tremor, a qPCR assay, designed based on the new sequencing data, was used to profile the presence of APPV across affected and unaffected piglets. The newly designed qPCR assay provided the required specificity for the diagnostic of the new APPV strain. Specifically, all the piglets exhibiting congenital tremor as well as their littermates were positive for APPV, while the litters not affected were negative. Profiled at the same time with piglets, all the dams were qPCR negative. The majority of the pigs (69.6%) were still positive for APPV at approximately 30 d of age. The clear signs of tremor disappeared in the vast majority of pigs at 50 d of age (91.6%) and a suggestive decrease in viremia was observed at this point ($P < 0.07$). Initial Nanopore sequencing data also suggested that the lack of specificity of the reported APPV qPCR assay (Arruda et al., 2016) was the most likely reason for the early negative APPV diagnostic analysis. Specifically, the forward primer of the reported qPCR assay includes 3 nucleotide mismatches compared to the sequences obtained from the newly identified strain.

In a subset of 171 piglets representing 25 litters affected by congenital tremor, a suggestive association was observed between APPV viral titer ($P < 0.15$) and congenital tremor; piglets exhibiting tremor displayed a larger viral titer in the blood (6.55 ± 0.21 log10 copies of APPV/mL of serum) compared to visually normal littermates (6.25 ± 0.21), suggesting a role of APPV viral load in the expression of congenital tremor. We hypothesize that the effects are actually larger since most of the preweaning mortality associated with congenital tremor occurred in the first 5 d of life, while the viral titer was measured in all available piglets at an average of 6.6 ± 0.3 d of age.

Genome Sequencing and Assembly of the Novel APPV Strain

Generating whole-genome sequencing data and complete genome assembly directly from clinical samples is challenging due to relatively low viral titer. In this study, we employed a whole-genome preamplification approach used successfully for generating a complete genome sequence of Zika and other viral genomes directly from clinical samples (Quick et al., 2017). The initial Nanopore sequencing reads of the novel strain and the complete genome of its closest APPV strain (MF167291.1) led to the design of 39 pairs of primers and overlapping amplicons expected to cover the entire genome. The majority of primer pairs led to successful amplification (92.3%), while combinations of adjacent primers were used to amplify the remaining regions. After a Nanopore sequencing run, base-calling using Albacore 2.3.3, and adapter removal using Porechop, there were 445,199 reads obtained. The reads were processed using bioinformatic scripts associated with Zika-pipeline (https://github.com/zibraproject/zika-pipeline). Using minimap2, 69.7% of the reads were aligned to the APPV strain (Ger-NRW_L277, MF167291.1). The obtained alignments were subjected to a script (align_trim.py) that removes primer sequences based on their coordinates and normalize the coverage. Following normalization and primer trimming, a subset of 36,166 sequences and the MF167291.1 reference sequence were used for variant detection using the “nanopolish variants” application. There were 618 Single Nucleotide Polymorphisms (SNPs) identified with an average of 52.6 SNPs/kb. A script (margin_cons.py) was used to filter out low-coverage and low-quality variants and generate a consensus sequence of the new strain. Compared to MF167291.1 coordinates, the consensus sequence lacked the first 60 nucleotides and had a gap of 217 nucleotides between 4,134 and 4,351 bp. A PCR

amplicon covering the gap was sequenced and the new genome was assembled (Acc. No. MK728876).

Phylogenetic Analysis of the APPV Strains

Complete genome sequences available in NCBI Nucleotide/Protein database consisting of 30 APPV strains isolated across the globe were subjected to phylogenetic analysis using the sequences of the viral genome and the viral polyprotein. Both analyses revealed 2 noticeably divergent clades: the large one contained 24 strains, from different geographical locations, while the smaller clade included 7 strains so far reported only in China (Figure 2.2; Figure 2.3). While some of the strains were isolated from asymptomatic piglets, strains from both clades were associated with the occurrence of congenital tremor (Postel et al., 2017; Zhang et al., 2017; Shen et al., 2018). The large clade showed limited clustering based on geographical location; in most cases, important diversity within regions was identified. The DNA sequence of the novel strain (MK728876) showed the highest similarity with a cluster of 3 APPV strains isolated from Germany (MF167290.1, MF167291.1, and LT594521.1), forming a strongly supported clade. All these 3 strains were recovered from piglets exhibiting congenital tremor (Postel et al., 2017). Similarities in this clade, at the level of DNA, varied from 97% to 98%. Both phylogenies found few affinities between the novel MK728876 strain reported here and the other U.S. strains (Figure 2.2; Figure 2.3). Clearly, NC_038964 and KR011347 strains are distantly related to MK728876 and KU194229, and the relatedness between the later strains is higher. Consequently, similarities ranging from 94% to 96%, were observed at the DNA level when the new strain was compared to the other 3 strains from

the United States. When the predicted polyprotein sequence consisting of 12 peptides was analyzed and compared across predicted viral peptides, the proportion of similarity between the new and a less similar U.S. strain (AKS24977) varied across nonstructural viral proteins ranging from 78.5% (NS5b) to 98.8% (NS4b). Using a short fragment of the nonstructural NS3 protein, Postel et al. (2017) showed similar clustering, emphasizing the widespread and the diversity of APPV.

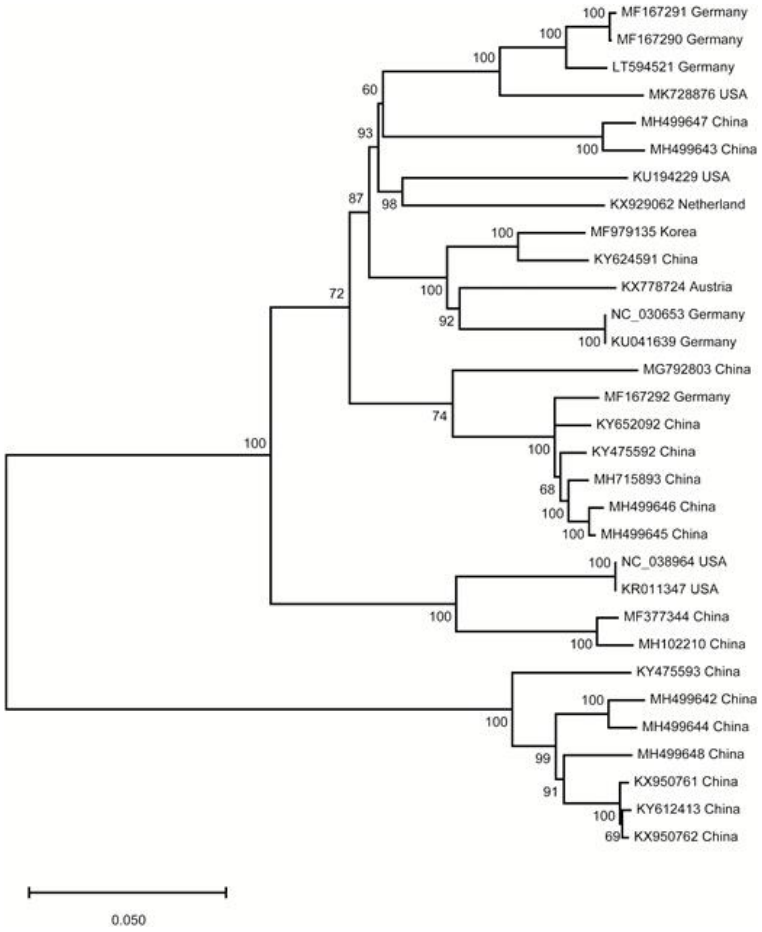

Figure 2.2. Phylogenetic analysis of the DNA sequences of APPV strains across the globe. The phylogenetic tree was generated by using the maximum likelihood method and Tamura-Nei model. The tree with the higher log likelihood is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 0.3303]). Analysis of the 31 sequences involved a total of 10,908 positions.

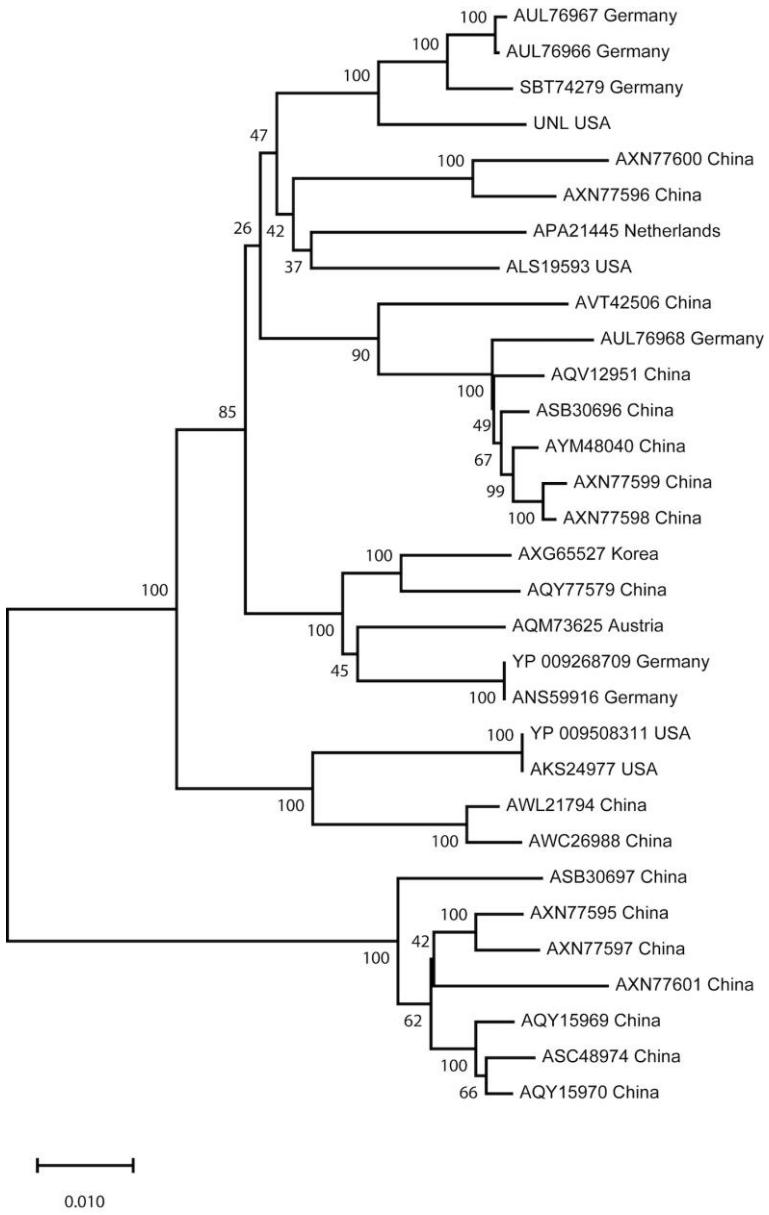

Figure 2.3. Phylogenetic analysis of the predicted polyprotein sequences of APPV strains across the globe. The phylogenetic tree was generated by using the maximum likelihood method and JTT matrix-based model. The tree with the highest log likelihood is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 0.2369]). Analysis of the 31 sequences involved a total of 3,642 positions.

The genome alignment of the various APPV strains indicates a high overall nucleotide diversity across the strains. The divergence is more evident in the 5' and 3' noncoding regions. However, substantial nucleotide polymorphisms are scattered across the viral polyprotein sequence. As a result, qPCR-based identification of all APPV strains could be challenging but the employment of degenerate oligonucleotides in qPCR assays or cost-effective metagenomic analyses could circumvent the divergence found among APPV strains.

In this study, we combined two approaches to detect and sequence the genome of a novel APPV strain. Preamplification of viral genome using random nanomers was employed for the initial sequencing data, while tilling amplicons were obtained and sequenced using partial sequencing data of the novel strain and the most-related and available APPV genome. Compared to the other methods, this approach is characterized by efficient enrichment of the low-abundance genetic material isolated from clinical samples and rapid turnaround in detecting a novel APPV strain. Low viral abundance is associated with a decrease in the likelihood of long RNA fragments being present in samples (Quick et al., 2017), and short-amplicon-based amplification, as employed in this report, alleviated this issue. As demonstrated in this study, qPCR-based detection has limited sensitivity when the targeted strain is highly divergent but, for highly similar families of viruses, the qPCR approach is the cost-effective strategy. While this approach is ideal for infections caused by single-clone viruses, it is less suitable for highly diverse or mixed viruses and strains. As shown here, data generated by amplicon-based sequencing is affected by incomplete and dropouts in coverage and lack of information at

both ends not covered by primers. Any approach involving preamplification is prone to contamination based on reagents/tissues used in previous laboratory analyses.

CONCLUSION

The presence of vacuoles in the white matter of the cerebellum, high preweaning mortality, and incidence of splayed legs were observed in newborn piglets affected by congenital tremor. Piglets affected by congenital tremor also had lower preweaning survival rates but larger birth weights compared to their unaffected littermates. Metagenomic analysis showed that APPV was the most abundant microbial species in the blood collected from piglets affected by congenital tremor. A qPCR assay, specific for the new strain, validated the discovery efforts based on metagenomic data, detecting the presence of APPV in all affected piglets and their unaffected littermates but absent from litters unaffected by congenital tremor. Piglets affected by congenital tremor exhibited higher APPV viral titer compared to normal littermates. Phylogenetic analysis of APPV strains across the globe revealed 2 highly divergent clades. Evidence of genetic similarity was found based on geographical location; also, important diversity within regions was observed. Variation of the APPV strains in the United States alone could lead to underdiagnoses of pestivirus-associated congenital tremors and emphasize the need for next-generation molecular diagnostic approaches based on robust, cost-effective, and real-time metagenomic analysis. Future studies of this family of swine *Pestiviruses* will need to provide critical information in areas such as mode of transmission, disease progression, variation in strain virulence, and the role of host genetics in disease susceptibility.

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CHAPTER 3: SEQUENCE DIVERSITY AND MOLECULAR DIAGNOSTICS OF APPV INFECTIONS

INTRODUCTION

Pestivirus is a genus within the *Flaviviridae* family. There are four major species within *Pestivirus*, with additional species being identified through novel sequencing approaches. With more viral species being discovered and fully sequenced, a diagnostic test should be able to identify any *Pestivirus* species. However, our previous research showed substantial diversity at the DNA and protein level among atypical porcine pestivirus (APPV) strains (Sutton et al., 2019) that could prevent qPCR based molecular diagnostic.

While the polyprotein sequences are characterized by important genetic diversity, the 5'-untranslated regions (5'UTR) of the viral genomes are conserved across pestivirus species (Deng & Brock, 1993; Hellen & de Breyne, 2007). In the 5'UTR, certain signals are modulating viral RNA replication, transcription, and translation (Deng & Brock, 1993; Tautz et al., 2015). Pestiviruses use host machinery to translate their genomes (Tautz et al., 2015). Pestiviruses have an internal ribosome entry site (IRES) instead of a 5' cap which is the basis of replication signaling. The IRES recruits host ribosomal units to the initiation codon of the viral genome for replication (Hellen & de Breyne, 2007; Tautz et al., 2015). Therefore, it is a critical part of the pestiviral genome that must be conserved for the virus to infect and propagate. This conservation makes the 5'UTR a candidate region for a diagnostic test to detect atypical porcine pestivirus (APPV) genomes in piglets affected by congenital tremor.

MATERIALS AND METHODS

Sample Collection

Blood samples from piglets experiencing congenital tremor were collected from six different farms from the Midwest (MW1 to MW6). The operations varied in size and purpose from small family farms to research and commercial populations. The genetic source of affected piglets varied from purebred lines (ex. Berkshire) to commercial maternal crosses involving Large White and Landrace. The number of samples from each site varied with Table 3.1 providing details. Serum was obtained by centrifuging whole blood samples at 2,400 x g at 4°C for 30 minutes. Serum samples were stored at -80°C until ready to be processed.

Atypical Porcine Pestivirus RNA Isolation and cDNA Generation

Viral RNA was isolated from serum obtained from piglets affected by congenital tremor. Extraction utilized the MagMAX-96 Viral RNA Isolation kit (Applied Biosystems) in a high-throughput manner using the MagMAX Express Magnetic Particle Processor (Applied Biosystems) following the manufacturer's protocol (Table 3.1). The viral RNA was eluted in 50 µL of elution buffer. The AM1836_v2 protocol, which comes preloaded on the MagMAX Express Magnetic Particle Processor (Applied Biosystems) machine, was used. The viral cDNA was generated using extracted viral RNA, random hexamers, deoxynucleotides, and Superscript III reverse transcriptase (Life Technologies).

Row Position		Reagents and Volume	
A	**Sample Well**	Bead Mix	20 µL
		Sample/Serum	50 µL
		Lysis/Binding Solution	130 µL
B	**1st Wash-1**	Wash Solution 1	150 µL
C	**2nd Wash-1**	Wash Solution 1	150 µL
D	**1st Wash-2**	Wash Solution 2	150 µL
E	**2nd Wash-2**	Wash Solution 2	150 µL
F	**Elution**	Elution Buffer	50 µL

Table 3.1 MagMAX viral RNA isolation plate setup for use with MagMAX Express Magnetic Particle Processor.

Oxford Nanopore Sequencing and Assembly of the APPV Midwest Site-2 Strain

A set of 22 pigs expressing congenital tremor and originating from Midwest Site-2 (MW2) tested negative for APPV using qPCR assays described in Arruda et al. (2016) and Sutton et al. (2019). Sequencing of APPV from MW2 was based on genome preamplification utilizing 39 pairs of primers covering the whole viral genome and Oxford Nanopore MinION sequencing as described in Sutton et al. (2019). Briefly, the overlapping primers covering the whole viral genome were designed using Primal Zibra (https://primal.zibraproject.org) and MF167291.1 and MK728876 (MW1) as genome reference sequences. Equal amounts of amplicons were pooled and purified using AMPure XP beads (1x ratio of cDNA to beads). The concentrations of DNA were determined using Qubit 1.0 fluorometer (Life Technologies). Library preparation and

Nanopore sequencing were based on manufacture's protocols using Ligation Sequencing kit 1D (SQK-LSK108) and the MinION (Oxford Nanopore).

Bioinformatic analyses and genome assembly of the FAST5 reads produced were based on tools initially designed for the Zika virus (https://github.com/zibraproject/zika-pipeline). First, adapters were removed from the obtained FAST5 reads with Porechop v0.2.3 (Nanopore). The reads were then mapped to the reference genome (MF167291.1) with minimap2 (https://github.com/lh3/minimap2) and converted to BAM format with "samtools view". The preamplification primers were trimmed and read coverage was normalized using the *align_trim.py* script. Sequence variants between the reference sequence and MW2 APPV genomes were called using "nanopolish variants" (https://github.com/jts/nanopolish). Finally, the consensus sequence of the MW2 genome was generated with the *margin_cons.py* script.

Atypical Porcine Pestivirus 5'UTR PCR and qPCR Testing

Primers for PCR amplification of the 5' UTR of APPV were designed based on the longest available genome sequences on the NCBI database (e.g., MH715893.1, KX929062.1). One forward and two reverse primers were designed in 5'UTR APPV regions characterized by high sequence conservation. Polymerase chain reaction (PCR) amplification of the 5' UTR of the viral cDNA and dideoxy sequencing was performed on samples collected from five Midwest sites that reported congenital tremor (MW1 to MW5). Sequences were analyzed and aligned with Sequencher software version 5.4.6 (Gene Codes Corporation).

A qPCR assay was designed targeting the conserved regions of the 5'UTR of the APPV genome using the sequences obtained from the different sites. The new assay (Forward: 5'-CCT GAG AGA GAG GTA CCG A-3'; Reverse: 5'-CCT CAG TAG ACC CTA CAC CTA G-3'; Probe: 5'-TG ACG TCT GC CCC GTA CTC G-3') was used with TaqMan Master Mix (Life Technologies) and the CFX384 Real-Time PCR (BioRad) instrument to detect the presence of APPV across sites.

Phylogenetic Analysis of Multiple Strains of APPV

Phylogenetic analysis of the APPV 5'UTR was based on the sequence alignment of five MW strains and 47 other available APPV strains. The latter sequences were retrieved from GenBank. the phylogenetic tree was obtained using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993) implemented in MEGA X (Kumar et al., 2018).

The 5'UTR was aligned across MW1 to MW5 and other full-length available sequences (15 APPV strains) using MULTALIN (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalinan.html). Conservation of the 5'UTR was assessed at each nucleotide site across these APPV strains. The same approach was performed for each of the twelve genes. The strains used in this analysis included MW1 (MK728876) and the 15 full-length available sequences on NCBI and analyzed in Sutton et al. (2019), except for N^{pro} for which MW1 N^{pro} sequence was incomplete.

RESULTS AND DISCUSSION

Variation in APPV RNA Sequence Led to Strain-Specific qPCR Assays to Detect APPV in Piglets with Congenital Tremor

Samples were collected from piglets exhibiting congenital tremor along with additional littermates from a family farm located in the Midwest (MW2). The samples were tested with two available qPCR assays (Arruda et al., 2016; Sutton et al., 2019). The samples were either APPV negative or the results were inconsistent between the assays. New PCR primers were designed to capture the region of the qPCR assay previously reported in Sutton et al. (2019) in order to assess sequence variation in MW2 samples. Sequence analysis in MW2 samples revealed three and two variations in the qPCR probe and reverse primer, respectively, compared to the assay reported in Sutton et al. (2019). These differences most likely affected the ability of the qPCR assay to detect the APPV genome in MW2 samples, leading to false-negative results. The sequence amplicon was utilized to create an MW2 strain-specific qPCR assay which was tested on all 22 MW2 samples. The APPV genome was detected in 19 of 22 samples with an average Cq value of 36.4 using this site/strain-specific diagnostic test.

Substantial Variation Across Viral Polyprotein Genes Could Lead to Challenges in Accurate Genetic Diagnostic of APPV in Piglets with Congenital Tremor

Using the same approach used in Sutton et al. (2019), Oxford Nanopore sequencing was employed to sequence a novel strain isolated from MW2 samples. There were 811 variations identified between this genome and the reference (MF167291.1). There were also 1,637 DNA variations between MK728876 (MW1) and MW2 using MULTALIN. These farm sites from where these samples originated were approximately

100 miles apart and yet had more differences between their sequences compared to the reference APPV genome isolated from Germany (MF167291.1). This highlighted the high degree of variation among APPV strains which can result in underdiagnosis of APPV induced congenital tremor.

Across multiple APPV genomes, the polyprotein genes exhibited a high level of genetic variation with an average of 68% nucleotide site conservation, with structural and non-structural genes being about the same (Table 3.3).

Gene	# of Strains	Gene Length	% Conserved
N^{pro}	15	540	66.85
Core Protein C	16	333	68.47
E^{rns}	16	630	68.73
E1	16	597	69.01
E2	16	723	66.67
p7	16	192	64.58
NS2	16	942	64.23
NS3	16	2061	72.15
NS4A	16	201	70.15
NS4B	16	1017	71.39
NS5A	16	1416	65.32
NS5B	16	2256	70.66
5’UTR (Global+MW)	20	299	83.33
5’UTR (Global)	15	299	85.00

Table 3.3. Results from MULTALIN comparing different APPV strains across 5’UTR and polyprotein genes.

Important Sequence Similarity in the 5'UTR was Observed Across APPV Strains

Sequence comparison across the 5'UTR and polyprotein sequences revealed that the 5'UTR is the most conserved across APPV strains, with nucleotide sites similarity ranging from 83% between global and Midwest site strains and 85% between the global strains only. This high level of conservation makes the 5'UTR an ideal candidate region for a universal diagnostic test as it would be able to detect more than one strain of APPV.

Additional samples from the other four Midwest sites (MW3-MW6) were obtained and processed as described above. The presence of APPV was tested using the previous two qPCR assays (Arruda et al., 2016; Sutton et al., 2019) and the MW2 specific assay. Results varied across the assays and sample sites. There was not one assay that could consistently detect the presence of the APPV genome within congenital tremor samples across sites. Using multiple assays on a single sample is time-intensive and costly. The need for a universal detection assay would reduce the time and resources needed to detect APPV outbreaks. While some diagnostic labs currently employ the use of degenerate probes and primers to have a greater chance of capturing multiple strains (Peddireddi, 2020), there are still limitations. Since the viral genome will continue to evolve even different combinations of degenerate primers could lead to spurious results in time.

The qPCR assay designed targeting the conserved regions of the 5'UTR of the APPV genome was tested on a subset of samples from each Midwest sites. The diagnostic test could detect APPV in all samples obtained from congenital tremor affect

pigs from all six Midwest sites (Table 3.2) contains a summary of samples used in qPCR testing.

Farm	# of Piglet Samples	# of Positive Samples From 5'UTR qPCR (Pigs with Congenital Tremor Tested)
MW1	399	2 (2)
MW2	22	2 (2)
MW3	7	3 (3)
MW4	2	2 (2)
MW5	17	2 (2)
MW6	3	3 (3)

Table 3.2. Piglets information from Midwest sites farms.

Despite the sequence similarity, phylogenetic analysis of the APPV 5'UTR across 52 global strains (including five MW strains) showed some similar clustering as previously observed (Sutton et al., 2019), when the whole viral genomes were used (Figure 3.1). For example, the small cluster of strains originated from China was still present and MW1 (MK728876.1) still clustered next to a strain originated from Germany (LT594521.1). There were also some signs of possible evolution at a regional level, with a clear example seen in a small cluster of strains originated from Switzerland. Also, despite the short distances between MW sites, some of these strains did not cluster together.

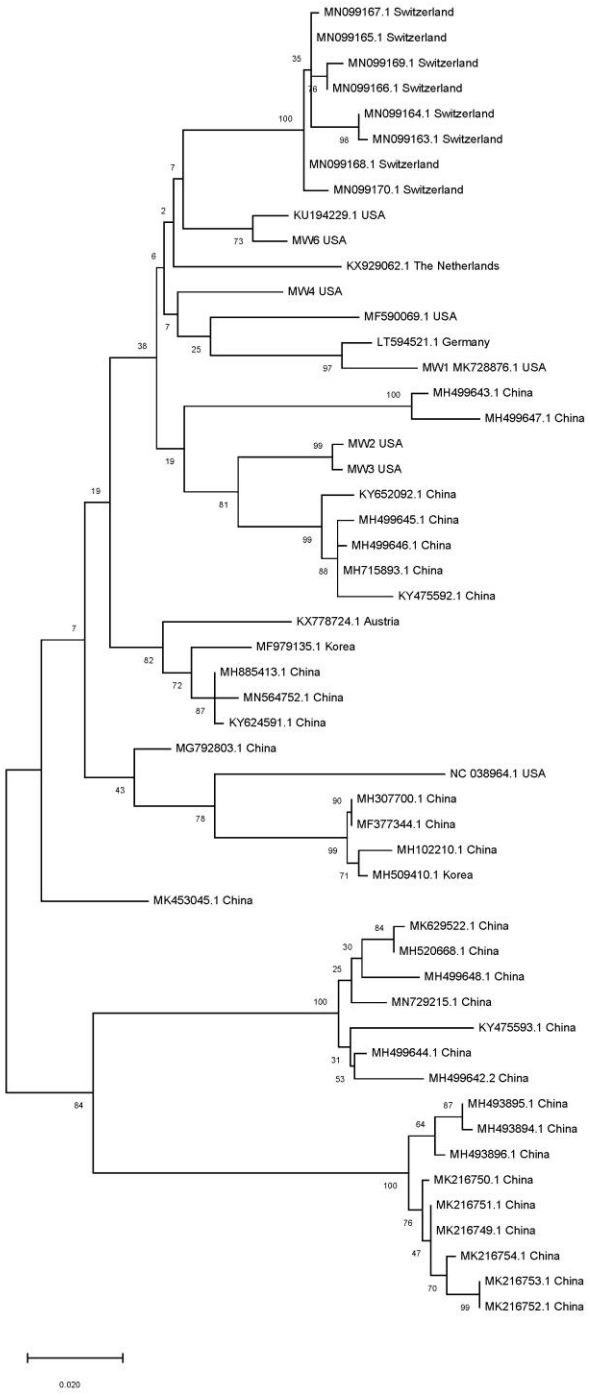

Figure 3.1. Phylogenetic analysis of Midwest Sites' 5'UTR sequences with additional available APPV strains.

CONCLUSION

The high degree of sequence variation observed in the coding genes of the APPV viral genome makes it difficult to use a single diagnostic assay to correctly estimate the prevalence of APPV. Following inconsistent qPCR diagnostic results involving samples collected from piglets affected by congenital tremor and originated from different farms, novel strains of APPV were sequenced from multiple sites across the Midwest. Substantial sequence variation across polyprotein DNA sequence prevented accurate diagnosis by qPCR leading to spurious results. Full-length sequence analysis of multiple APPV strains determined that the 5'UTR had a high level of conservation, making it an ideal candidate region for qPCR diagnostic assays. A newly designed qPCR assay targeting the 5'UTR of APPV was able to detect APPV in piglets affected by congenital tremor across six Midwest farms. As the APPV genome continues to evolve, the qPCR assay will potentially need to be reevaluated to ensure the nucleotide sites are still conserved for accurate detection of APPV.

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CHAPTER 4: EFFECTS OF *DQB1* HAPLOTYPES ON APPV INFECTION

INTRODUCTION

The swine leukocyte antigen (SLA) complex plays an important role in the immune response to viral pathogens and vaccines. This gene-dense region of SSC7 is separated into three regions designated class I, II, and II (Gustafsson et al., 1990; Kanai et al., 1999; Chardon et al., 2000; Smith et al., 2005; Renard et al., 2006; Ho et al., 2009; Lunney et al., 2009; Ho et al., 2010; Park et al., 2010; Minh Thong et al., 2011). The genes in the SLAII region, which is highly homologous to the human major histocompatibility complex (MHC) class II, are involved in antigen presentation of exogenous peptides to CD4+ helper T-cells during infections. The protein binding antigens, SLA-DR and SLA-DQ, are expressed on the surface of macrophages, B cells, and other antigen-presenting cells (Gustafsson et al., 1990; Chardon et al., 2000; Smith et al., 2005; Ho et al., 2009; Lunney et al., 2009; Ho et al., 2010; Minh Thong et al., 2011).

The SLAII region has been associated with differential response to viral pathogens through genome-wide association studies. Walker et al. (2019) observed variation in levels of porcine respiratory and reproductive syndrome virus (PRRSV) specific antibodies in infected pigs which was partially explained by a major quantitative trait locus (QTL) near the SLAII region. Additionally, another QTL mapped near the SLAII region that partially explained variation in porcine circovirus type 2 (PCV2) viral load (Walker et al., 2018).

Within the SLAII region, there are two SLA-DQB genes: *SLA-DQB1*, which is the functional gene encoding β chains, and *SLA-DQB2*, a pseudogene. The *SLA-DQB1*

gene contains a high degree of polymorphisms which can hinder attempts to sequence the gene as a whole (Kanai et al., 1999; Chardon et al., 2000; Renard et al., 2006; Lunney et al., 2009; Ho et al., 2009; Ho et al., 2010).

Atypical porcine pestivirus (APPV) is a virus recently discovered across the world. It has been identified as a potential cause of congenital tremor type A-II. Affected newborn piglets have difficulties nursing and can lead to malnutrition and death from starvation (Zhang et al., 2016; Schwarz et al., 2017; Dessureault et al., 2018; Zhang et al., 2018). In a controlled APPV-based antigen exposure of gilts (n = 91), 45.1% of the females generated litters affected by congenital tremor while the rest generated normal litters (Sutton et al., 2019). This response could be a result of a less efficient viral exposure, but we cannot rule out the potential role of host-genetics in viral disease progression. Since the *SLAII* region was previously involved in multiple viral diseases in pigs, we hypothesize the SLAII locus, and specifically *DQB1* haplotypes, could also influence APPV susceptibility in pigs.

MATERIALS AND METHODS

Sample Collection and Isolation of Genomic DNA

An APPV-based controlled exposure experiment was conducted by subjecting maternal crossbred gilts (n=91) to an inoculate obtained from fetal fluids and membranes collected at farrowing from litters affected by congenital tremor (Sutton et al., 2019). Ear notches or tail clippings were collected from the gilts for DNA isolation. These gilts were inseminated with commercial Duroc semen. Litter size traits, including total number of piglets born (TNB), number of mummified and stillborn, and incidence of congenital

tremor and splayed legs were recorded at farrowing. The weight of the piglets was measured at birth and at weaning. Mortality was monitored daily until weaning.

Genomic DNA was extracted from the tissue of dams using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's protocol except tissues were lysed over-night and DNA was eluted in 50 µL of Buffer AE after incubating on spin column for 5 minutes at room temperature. DNA quality was determined with the Qubit fluorometer (Thermo Fisher Scientific) and Qubit dsDNA BR Assay kit (Thermo Fisher Scientific). Samples were then diluted to a working concentration of 15 ng/µL with RNase-free water.

DQB1 Amplification, Sequencing, and Haplotype Assignment

The *SLAII* haplotypes of dams were based on *DQB1* haplotypes of specific genomic segments of the *DQB1* gene. These segments were amplified by polymerase chain reaction (PCR) using GoTaq Flexi DNA polymerase (Promega). Amplicons were checked for correct product size through gel electrophoresis and then sequenced using dideoxy sequencing. These sequences were compared to full-length reference *DQB1* haplotype sequences (Immuno Polymorphism Database, https://www.ebi.ac.uk/ipd/mhc) to obtain individual haplotypes.

Statistical Analyses

The relationship between the occurrence of congenital tremor across litters and the presence of a specific sow *DQB1* haplotype was evaluated by Pearson Chi-Squared Contingency Test. The potential effects of the sow *DQB1* (SLAII) haplotypes on the

average pre-weaning mortality and the incidence of congenital tremor were estimated as haplotype substitution effects. Contrasts between haplotypes were estimated using a linear model with values 0, 1, and 2 corresponding to the sow having 0, 1, or 2 copies of the assigned haplotypes. The linear model also included litter size (Total Number Born) as a covariate.

RESULTS AND DISCUSSION

DQB1 Haplotype Profiling in Sows Affected by Congenital Tremor

In a previous controlled APPV-based antigen exposure of gilts (n = 91), we found an important variation in terms of litters affected by congenital tremor, piglet pre-weaning survival, and incidence of splayed legs (Sutton et al., 2019). Specifically, only a fraction of the exposed gilts produced litters affected by congenital tremor (45.1%). The piglets affected by tremor had a substantially higher incidence of splayed legs (33.0 % vs 0.8 %) and pre-weaning mortality rates (46.4% vs 15.3%) especially in the first days of life compared to unaffected piglets. As expected, piglets affected by congenital tremor exhibited higher APPV titer ($P < 0.15$) and larger birth weights ($P < 0.05$) compared to unaffected littermates.

The SLAII haplotype profiling based on the highly polymorphic *DQB1* gene uncovered 11 *DQB1* haplotypes in 83 of 91 gilts successfully genotyped (Figure 4.1a). With the exception of the *DQB1-0901* haplotype which was only observed in the subpopulation of sows who produced unaffected litters (Figure 4.1c) the rest of the haplotypes were observed in both affected and unaffected litters with congenital tremor (Figure 4.1b and c). Out of the 11 *DQB1* haplotypes identified, there was one novel

haplotype determined which was not part of the reference database (https://www.ebi.ac.uk/ipd/mhc). The novel haplotype (*DQB1-UNL1)* was observed in both groups of sows who had affected litters or unaffected litters. While it was not a major haplotype (>10% frequency), it was observed at a higher rate in sows producing affected litters than those having unaffected litters (7.9% vs 2.22%). Four major haplotypes were observed in both phenotype groups: *DQB1-0202* (35.56% in dams with unaffected litters, 34.21% in dams with affected litters), *DQB1-0203* (12.22%, 11.84%), *DQB1-070101* (13.33%, 10.53%), and *DQB1-070102* (12.22%, 14.47%).

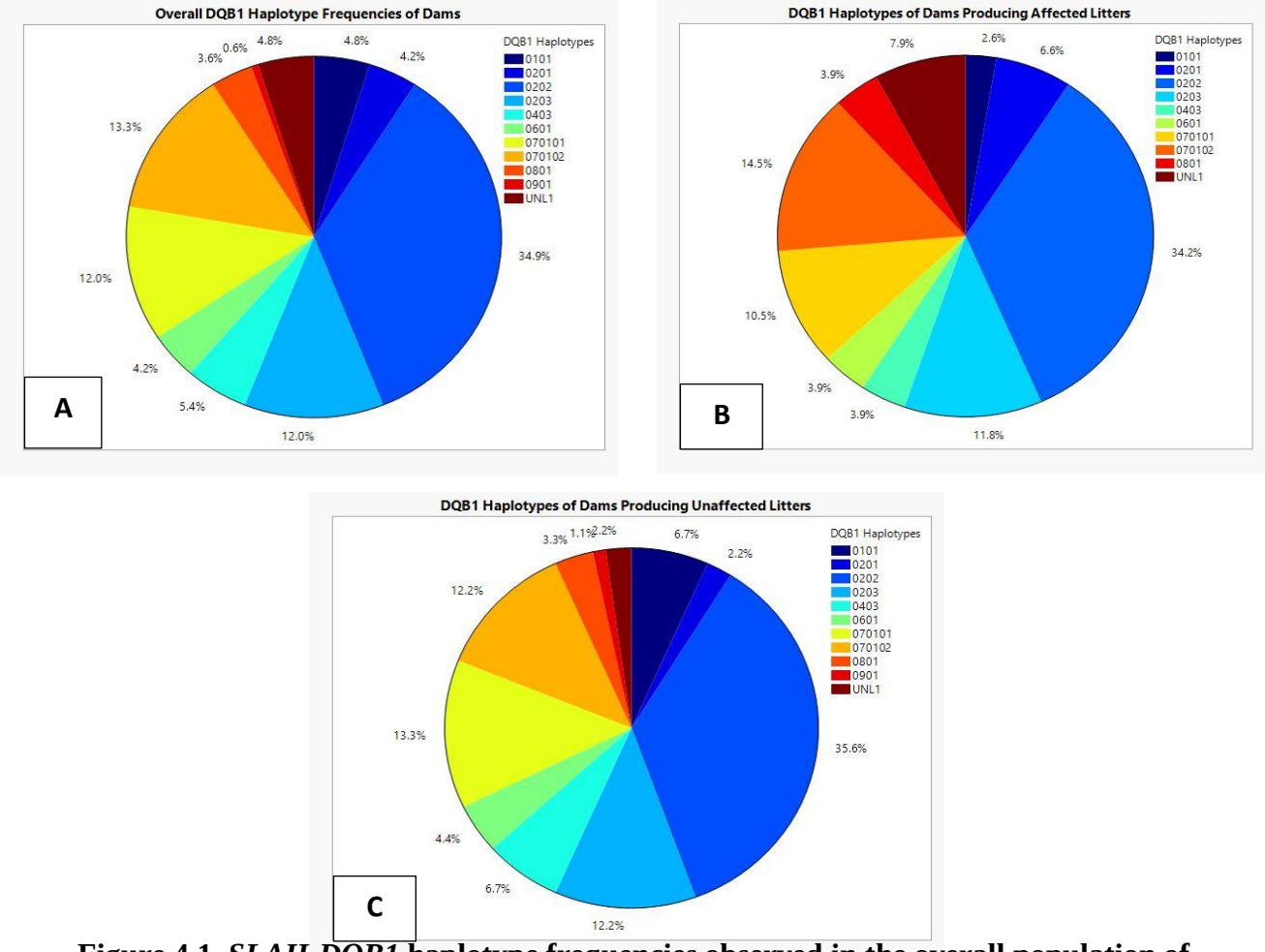

Figure 4.1. *SLAII-DQB1* haplotype frequencies observed in the overall population of dams profiled (a), in dams who generated litters affected with congenital tremor due to APPV (b), and in dams generating unaffected litters (c).

Considering all the dams, despite the phenotypic status of their litter, there was a 66.3% level of heterozygosity observed based on *DQB1* haplotypes. When litter status was taken into account, sows generating unaffected litters had a slightly higher level of heterozygosity (37.78%) than those who had litters affected by congenital tremor (28.95%). The two most predominated heterozygotes observed in both sets of dams were *0202/0203* (15.56% unaffected, 15.79% affected) and *070101/070102* (15.56%, 18.42%). Meanwhile, the predominate homozygote, *0202/0202*, was similar in sows with unaffected litters (26.67%) and those with affected litters (26.32%).

No significant relationship was observed between the occurrence of congenital tremor and the presence of specific *DQB1* haplotypes ($P > 0.08$). A suggestive relationship was observed between the presence of haplotype *DQB1-UNL1* and the occurrence of congenital tremor (Pearson $Chi^2 = 2.89$, $P > 0.09$). While this haplotype is a relatively minor haplotype across the dataset, *DQB1-UNL1* exhibited the largest frequency difference (±5.67%) between sows with affected (7.9%) and unaffected litters (2.22%). Additionally, no significant relationship was observed between the presence of congenital tremor and the sow *DQB1* haplotype homozygosity or heterozygosity (Pearson $Chi^2 = 0.719$, $P > 0.39$).

Effect of *DQB1* Haplotypes on Pre-Weaning Survival

Haplotype substitution analysis demonstrated that *DQB1*-based *SLAII* haplotypes in sows do not appear to influence the incidence of congenital tremor in their piglets ($P > 0.83$). A potential suggestive effect of *DQB1* haplotypes was observed on pre-weaning mortality ($P > 0.07$), a phenotypic variation driven by APPV infections that are leading to

congenital tremor. Two of the haplotypes identified, *DQB1-070101* (12.1% overall frequency) and *DQB1-0801* (3.6%) were associated with a reduction in piglets pre-weaning mortality while *DQB1-070102* (13.3%) was associated with an increase in pre-weaning mortality compared to the average haplotype effects ($P < 0.05$). The effect of the sow *DQB1* haplotypes on APPV viral titer in piglets was not conducted since a large portion of piglets affected by congenital tremor did not reach five days of age (76%), the time of the blood collection for viral titer.

CONCLUSION

Host genetics have been known to influence some aspects of viral disease susceptibility and severity. The *SLAII* region of the swine genome is involved in antigen presentation during viral infection. In this region, the *DQB1* gene encodes β chains used to present antigens on cell surfaces. A genotype-by sequencing protocol was designed and implemented to assign *SLAII* haplotypes to sows subjected to an APPV-based antigen exposure that led to litters affected by congenital tremor. Most of the *DQB1* haplotypes were identified in both phenotypic groups, specifically in sows that generated litters that were either affected or unaffected by congenital tremor. While there was no significant relationship between the presence of a particular sow *DQB1* haplotype or *DQB1* homozygosity/heterozygosity and the incidence of congenital tremor, a suggestive relationship was detected between the presence of a novel haplotype in sows (*DQB1-UNL1)* and congenital tremor in piglets. A suggestive effect of the dam's *DQB1* haplotype was observed on the litter pre-weaning mortality. However, the limited number of samples and the lack of normal distribution of the targeted traits could impact the

outcome of this analysis. In addition, the variation in response across sows could be also a result of a less efficient APPV exposure. There is no evidence that sows became viremic following experimental APPV exposure. Additional more detailed research would need to be conducted to better assess the host genetics role in APPV susceptibility and severity of infection.

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