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Characterization of strain PTV-2 USA/IA65463/2014 and strain PTV-11 USA/IA09592/2013 of Teschovirus A: Experimental inoculation, distribution of nucleic acids and development of Teschovirus encephalomyelitis

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Characterization of strain PTV-2 USA/IA65463/2014 and strain PTV-11 USA/IA09592/2013 of *Teschovirus A*: Experimental inoculation, distribution of nucleic acids and development of Teschovirus encephalomyelitis

by

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The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

To my wife Victoria, and my sons Francisco and Pietro. To my family, Elba, Hector, Cecilia and Gisela and my wife's family. Thank you for your unconditional support.

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ABSTRACT

Teschovirus A (previously *Porcine teschovirus;* PTV) is the etiologic agent of Teschovirus encephalomyelitis (TE). Historically, PTV-1 has been recognized as one of the most pathogenic serotypes, although other serotypes have been described to cause TE. Hence, most information about the pathogenicity, epidemiology and control of disease relies on data from studies involving PTV-1.

In recent years, two cases submitted to the Iowa State University Veterinary Diagnostic Laboratory with a history of polioencephalitis and/or myelitis were identified and genomic sequencing characterized these isolates as (sero)types PTV-2 and PTV-11.

Although multiple serotypes have been identified and genetically characterized, the neuropathogenicity of some of these serotypes has not been fully elucidated.

To assess these isolates, we developed an experimental model to determine the neuropathogenicity of the PTV-2 and PTV-11 isolates and observed that both isolates caused histological lesions and clinical disease consistent with TE in cesarean-derived colostrum-deprived pigs. Furthermore, PTV RNA was detected in different tissues, serum and feces in all inoculated animals by RT-qPCR.

The experimental approach used in this research permitted to develop a successful platform to induce clinical disease. This is the first description of a neuropathogenic PTV-11 strain in the U.S. and the first experimental inoculation using a PTV-2 autochthonous U.S. strain after the initial description of PTV-2 strain O3b made by Long *at al.* in 1966. This is also the first assessment of the viral shedding, viremia and distribution by real time RT-

qPCR of nucleic acids of PTV-2 and PTV-11 in experimentally infected pigs with Teschovirus encephalomyelitis.

CHAPTER 1. INTRODUCTION.

Objective

Teschovirus A (previously *Porcine teschovirus;* PTV) is the etiologic agent of Teschovirus encephalomyelitis (TE). The species *Teschovirus A* includes thirteen different known serotypes and is the sole member of the genus *Teschovirus*, family *Picornaviridae*[1]. *Teschovirus A* is a single-stranded, linear, non-segmented positive sense RNA virus. The virus possesses a 28-30 nm icosahedral cáspside enclosing its genome. The genome is approximately 7.8 kb and encodes a single mRNA transcript. Only one open reading frame is described, encoding a polypeptide that is cleaved post-translationally in the different structural and non-structural proteins.

The most severe forms of Teschovirus encephalomyelitis is associated with *Teschovirus A* serotype 1 (PTV-1). The virus is ubiquitous and has a worldwide distribution affecting commercial and feral swine populations. In the U.S., the presence of PTV-1 strains have been documented in Minnesota; however, outbreaks of clinical disease are only sporadically reported. Outbreaks of neurological disease associated with neuropathogenic strains of the virus other than PTV-1 have been more widely and frequently reported.

To further advance our understanding of TE; the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) has pursued the isolation of *Teschovirus A* associated with clinical cases. Two isolates were identified as *Teschovirus A* serotype 2 (PTV-2) and *Teschovirus A* serotype 11 (PTV-11).

The main objectives of this thesis were two-fold 1) develop an experimental model for *Teschovirus A* and 2) investigate the neuropathogenicity of *Teschovirus A* serotypes 2 and 11 through the reproduction of clinical signs, evaluation of the presence, severity and distribution of histopathologic lesions in central nervous system (CNS) tissue and detection of *Teschovirus A* RNA in multiple samples types from colostrum-deprived cesarean-derived pigs with TE.

Thesis Organization

This thesis consists of six different chapters organized as: general introduction, literature review, epidemiological background information from ISU-VDL, research submitted for publication in a peer reviewed journal (Viruses); preliminary results from an experiment involving the detection PTV nucleic acids and a final general summary.

Statement of the Problem

Recently outbreaks of neurologic disease are being observed more frequently in association with the detection of *Teschovirus A* by polymerase chain reaction (PCR) assays at the ISU-VDL. Affected animals submitted from different swine systems and epidemiological information provided by submitting veterinarians indicates that outbreaks are affecting more animals, from different age ranges and lasting longer in affected herds than observed previously. Economic losses in affected herds can be significant and currently there are no efficient methods to prevent or treat the disease. PTV strains not previously described appear to be emerging in addition to strains that have traditionally predominated.

Strains of PTV are endemically distributed within commercial swine operations and majority of growing and adult pigs have detectable levels of *Teschovirus A* antibodies; although, the association of serological results with the prevalence and severity of clinical disease caused by *Teschovirus A* infection is poorly understood. Hence, from a swine medicine standpoint, it is essential to determine whether the isolates PTV 2 and PTV 11 are neuropathogenic and capable of inducing clinical disease.

CHAPTER 2. LITERATURE REVIEW.

Introduction

Teschovirus A (PTV) has been isolated throughout the world, and swine are the only natural known host for the virus [2]. *Teschovirus A* is the causative agent of TE and may contribute to reproductive, respiratory, enteric, and dermatological clinical conditions [3]. A majority of infections are asymptomatically; and symptomatic presentations are thought to occur sporadically[2,4]. The virus is consistently found in feces from all age categories [5]. *Teschovirus A* consists of thirteen different serotypes [6]. The pathogenicity of all these serotypes have not been fully assessed, serotypes can be further divided in different biotypes that can be either pathogenic or non-pathogenic.

Taxonomy and Classification

Teschen disease was originally reported in 1929 by MVDr. Leopold Trefny in the district of Teschen, Czechoslovakia, as a highly virulent fatal disease of swine [7]. Shortly thereafter the disease was called "encephalomyelitis enzootica suum non purulenta," and the etiologic agents was identified as "*Teschen virus*" [8,9]. The disease has also been known as "Koblouck's disease" and "Teschen disease". Similar clinical signs were noted in the United Kingdom (1957) and Denmark (1959) where the disease was identified as "Talfan disease" and "benign enzootic paresis", respectively[10,11]. Also during 1959, the virus was identified to belong to the "enterovirus group of swine [sic]" [12] and the FAO/WHO/OIE Animal Health Yearbook introduced the eponym "Teschen disease" and made the disease internationally notifiable [13].

Eight distinctive serotypes were identified by different authors by serum neutralization tests [14-21] and three distinctive cytopathic effects (CPE) were consistently noted in tissue culture[22] .

Hence, a new classification for *porcine enteroviruses* was proposed and strains were divided depending upon the cytopathic effect exhibited in group CPE I, group CPE II and group CPE III. This allowed differentiation of strains into separate groups and permitted a more stringent classification.

In the year 1971 the International Committee on Taxonomy of Viruses (ICTV) (formerly known as the International Committee on the Nomenclature of Viruses) introduced the genus *Enterovirus* and a scientific criterion was established for the introduction of new viruses to this genus [23]. In 1974, the ICTV introduced the term "*porcine enteroviruses*" as a new member of the genus *Enterovirus* [24], and isolates such as Talfan and Teschen and other strains of the virus were included in this genus. In 1979, three new serotypes were identified, thus the number of prototype strains increased to eleven [25]. In 1994, two new prototype strains were identified increasing again the number of prototype strains (serotypes) from eleven to thirteen [26].

In summary, the *Porcine enteroviruses* (PEVs) were classified into 13 distinct serotypes (PEV 1–13) that included antigenically related strains of the virus with presumably different levels of virulence. Due to their distinct characteristics, they were classified as group I (PEV serotypes 1–7 and 11–13), group II (PEV-8), and group III (PEVs 9 and 10). The classification into distinctive serotypes is based on the criteria established by Dunne *et al*. where the cross-neutralization reactivity of a serum should have a minimum reaction of 5% of the homologous serum titre to be defined a standalone serotype. The literature describes minimal cross protection across serotypes *in vitro* (i.e. No cross-reaction above 5% of homologous titre); although, there is

evidence of reciprocal cross neutralization between different strains of the same serotype (i.e. cross-reaction above 5% of homologous titre) [27,28].

In 1999, the genetic cluster composed by the *Porcine enterovirus 1* strains was renamed as *Porcine teschovirus* and the genus *Teschovirus* was created. This change in nomenclature occurred during the XIth International Congress of Virology held in Sydney, Australia. The genus name was derived from the disease name (Teschen disease) and included all strains of *porcine enterovirus 1* known to that date [29]. In the 7th ICTV report, the remaining serotypes (i.e. PEVs 2-7 and 11-13) were abolished and *porcine enterovirus 8* was reclassified as *porcine enterovirus A.* Additionally, both *porcine enterovirus 9* and *porcine enterovirus 10* were renamed as *porcine enterovirus B*; however, *enterovirus A* and *B* remained under the *Enterovirus* genus [30,31].

In 2001, with the advent of more advanced techniques such as nucleotide sequencing and phylogenetic analysis a new classification of the members of the genus *Teschovirus* was proposed. In addition to *Porcine teschovirus 1*, PEVs in the CPE group 1 (i.e. serotypes 2–7 and 11–13) were renamed as *Porcine teschovirus* 1-11.

In 2011 and 2012, two new serotypes were identified in Spain and Hungary, increasing the number of serotypes to a total of thirteen (i.e. Porcine teschovirus A 1-13).

In 2014, the species *Porcine teschovirus* was renamed as *Teschovirus A;* although, the common name remained *Porcine teschovirus,* thus helping to differentiate it from the species name (i.e. *Porcine teschovirus* 1-13).

Virology

Teschovirus A is a linear, monopartite, single stranded ribonucleic acid (RNA) virus. The virus is non-enveloped with a spherical morphology of icosahedral symmetry and a diameter of 25-30 nm. The capsid shows a T=pseudo3 icosahedral structure composed of 60 protomers constituted by three surface polypeptides (i.e. VP1, VP2, VP3) and an internal polypeptidic component (i.e. VP4). *Teschovirus A* contains a genome of approximately of 7.0-7-2 kb which encodes a polyprotein flanked by a 5'- and a 3'- untranslated region (UTR). The single stranded RNA is enclosed in the capsid. The 5'UTR is preceded by a viral protein (VPg) and the 3' UTR is polyadenylated. The genome organization can be expressed as:

VPg+5'UTR$^{IRES\text{-}IV}$[L/1A-1B-1C-1D-2A^{npgp}/2B-2C/3A-3B^{VPg}-3C^{pro}-3D^{pol}]3'UTR-poly(A)

[32]

Where the protein VPg acts as a primer after being uridylated (VPg-pUpU) for the RNA-dependent RNA polymerase (3D^{pol}) to initiate RNA synthesis[33].

Located in the 5' UTR, there is a distinctive region of RNA necessary for the recruitment of the 40s-ribosome subunit, the internal ribosome entry site (IRES).

Teschovirus A possess an IRES type IV which uses most canonical translation initiation factors, less eIF4E which is cleaved by the 2A protease, and requires additional factors for efficient translation (ITAFs- IRES trans-acting factors)[34] to translate a single open reading frame (ORF).

Downstream the genome is encoded by a leader protein (L), which the function is unclear, and by the P1 conserved structural domain encoding the cáspside proteins 1A-1B-1C-1D (i.e. VP4, VP2, VP3, VP1).

The genome is followed by the P2 nonstructural domain encoding 2A, 2B and 2C. The 2A section of the genome translates a self-cleaving peptide which induces "ribosomal skipping" of the sequence resulting in the co-translational cleavage of the polyprotein. This cleavage is indicated to occur at the consensus sequence *NPG↓P*[35,36]. The function of the 2B non-conserved protein is unknown but analog poliovirus 2B protein is known to alter membrane permeability by the formation of pores.[37] The 2C section encodes an ATPase.

The P3 nonstructural domain encodes 3A, 3B, 3C and 3D. The function of 3A is unknown and 3B encodes a VPg that is linked to the 5'UTR. The 3C section encodes a protease (chymotrypsin-like proteinase) and the 3D section encodes the RNA-dependent RNA polymerase (RdRp).

The reference prototype strains recognized by the ICTV Picornaviridae Study Group and OIE are the following: PTV-1: Zabreh (Teschen) and Talfan strains, PTV-2: T80 strain, PTV-3: O2b strain, PTV-4: PS36 strain, PTV-5: F26 strain, PTV- 6: PS37 strain, PTV-7: F43 strain, PTV- 8: UKG/173/74 strain, PTV- 9: Ger-2899/84 strain, PTV-10: Ger-460/88 strain and PTV-11: Dresden strain. The last serotypes identified include PTV 12: CC25/SPA/2006 and PTV-13: wild boar/WB2C-TV/2011/HUN [3].

The different serotypes of *Teschovirus A* conceptually exist in nature as quasispecies. Vignuzzi *et al*. stated that "Quasispecies arise from rapid genomic evolution powered by the high mutation rate of RNA viral replication. Although a high mutation rate is dangerous for a virus because it results in nonviable individuals, it has been hypothesized that high mutation rates

create a 'cloud' of potentially beneficial mutations at the population level, which afford the viral quasispecies a greater probability to evolve and adapt to new environments and challenges during infection"[38]. Wang *et al*. indicated that a PTV-2 strain found in China emerged from the genetic recombination of a diverse parental antecessor sharing a high similarity with serotypes 2 and 5; and evolution of PTVs, as shown in other genera of picornaviruses, could be due to homologous recombination [39].

Immunity

Generally, infections caused by PTV are asymptomatic; although, seroconversion has been documented [15,40-42]. A weak and localized *in vitro* cellular mediated response against this pathogen was demonstrated in an experimental study; however, it appeared that the antiviral activity by cellular components do not play a major role in natural infections[43].

Animal age appeared to be an important risk factor when considering the likelihood of development of clinical disease; mature animals seemed to be less susceptible than younger animals [44]. Additionally, the quality and/or amount of colostrum intake has been associated with the development of clinical disease [44-47]. Piglets may absorb specific IgG and IgA against PTV if the mother had been previously exposed to the agent [48,49].

The secretion of specific IgA antibodies into the intestinal lumen is an important element of mucosal immunity; although, a defective response can occur if the number of viral particles exceeds the neutralizing capacity of the IgA present at the lumen of the intestine [50].

Experimental oral inoculation with strain T80 of PTV-2 demonstrated that IgA is the predominant antibody secreted into the intestinal lumen although newborn piglets rely on IgA

from milk until approximately 3 weeks of life, given that adequate intestinal production of secretory IgA does not occur until that time[51].

Pogranichniy *et al*. demonstrated through a prospective longitudinal serological investigation that colostrum neutralizing antibodies declined rapidly in a herd endemically infected by PTV, and by the 3rd week of age, piglets had minimal or no neutralizing antibodies in serum. [52].

Derbyshire *et al.* demonstrated the protective effect of maternal antibodies against PTV trough the experimental inoculation of PTV to a small herd. Piglets born from exposed or antibody-positive sows, that were subsequently challenged with PTV, excreted the virus in feces only after weaning when maternal lactogenic immunity is no longer available or colostral IgG in the blood has declined. In addition, piglets developed an antibody response during the post-weaning period when they were no longer protected by maternally derived immunity [40]. These findings suggest mucosal IgA may play a preponderant role against *Teschovirus A* infections, protecting weaning piglets through the intake of maternal antibodies or at post-weaning, after a primary immune response has been induced in the digestive tract [53,54].

Another study demonstrated that neonatal colostrum deprived germ-free piglets inoculated with a live avirulent strain of *Teschovirus A* will mount an immunological response approximately 5 days post-inoculation and this response progressively increased until the end of the experiment (15 days post-inoculation) [41].

A study investigating the antiviral activity of interferon alpha (IFN-α) and interferon beta (IFN-β) against PTV-2 infection demonstrated that IFN-α was more efficient than IFN-β when combating infection *in vitro,* indicating that innate immunological responses could play a significant role in preventing natural infections [55].

Experiments involving immunosuppressed animals have shown increased susceptibility to disease manifested by the exacerbation of clinical signs when compared to immunocompetent animals. In one experiment, pigs infected with PTV and treated with immunosuppressive doses of dexamethasone showed earlier and more severe clinical signs in addition to more severe histopathologic lesions when compared to non-dexamethasone treated pigs. There was also a positive correlation between the presence of severe lesions and dexamethasone-treatment when compared with the non-treated animals[56].

In a similar study, pigs infected with the T80 strain of PTV-2 and later treated with immunosuppressive doses of cyclophosphamide showed clinical signs compatible with TE. No clinical disease in uninfected and untreated control animals was observed. The authors indicated that infected cyclophosphamide-treated animals failed to develop a specific immune response in comparison with control animal [57].

Various authors have identified co-infections of *Teschovirus A* with other viruses. Authors have described clinical disease caused by PTV in association with *Porcine Circovirus 2* (PCV2), a virus known to induce immunosuppression in affected animals [58-61]; however it is known that PCV2 is endemic within most commercial herds and potential synergism between these two pathogens should be investigated further. *Porcine Reproductive and Respiratory Syndrome* virus (PRRSv) has also been identified in conjunction with *Teschovirus A* in several outbreaks of disease, however the interaction between these two viruses has not been fully assessed [60]. Co-infections produced by *Sapelovirus A, Enterovirus G,* and different strains of PTV have been documented but not fully assessed [5,62-64].

The coinfection of rotaviruses with low-pathogenic strains of PTV had been documented although no manifestation of clinical TE was observed [65,66]. No data is available for the joint effect of pathogenic strains of PTV and rotaviruses.

Pathogenesis

Entry routes

The fecal-oral route is the most established route of transmission; however, aerogenous, venereal and iagtrogenic routes have been postulated.

Fecal-oral route

The most relevant transmission route for PTV is the fecal-oral route[4,42,67]. After ingestion, the virus replicates in the tonsils and in different segments of the gastrointestinal tract[68]. The large intestine and ileum appear to be more prone to primary replication when compared to other segments of the gastrointestinal tract; although, viral replication has been demonstrated in other sections of small intestine. Viral replication appears to be more efficient and consistent within the large intestine when compared to other segments of the small intestines; although, there might be differences across serotypes[49]. The exact cells in which the virus attaches and initiate replication remains unknown. It is postulated that M cells are the primary target in which the virus attaches and is internalized by endocytosis; however, others have proposed that enterocytes also play an important role in this process.

The replication in the tonsil, suspected to occur primordially in the tonsillar follicles, could be later followed by retrograde axonal transport of virions to the CNS via cranial nerves Trigeminus, Facial and Glossopharyngeal[69].

Intranasal and respiratory route

Different studies have successfully induced clinical disease by intranasal inoculation [42,70-72]. Analogously, experiments with Poliovirus shown successful initial replication in the nasal cavity. The proposed entry route of viral particles is through the infection of epithelial cells. The initial replication of the virus in these cells could initiate a viremic phase and dissemination via retrograde axonal transport by Olfactory, Trigeminal and Facial cranial nerves has been theorized. The infection by direct deposit of viral particles in the pulmonary parenchyma and/or in lower sections of respiratory tract is theoretically possible; although, studies investigating solely this alternative are not available [73].

Venereal route

Teschovirus A has been successfully introduced into the genital tract of sows by artificial insemination via contaminated semen [74]. The presence of viral particles in urine has been documented and it can be as high as one thousand viral copies per microliter of urine [75]. Shedding of the virus during ejaculation is theoretically possible; however, natural infection by coitus have not been demonstrated. Evidence of PTV antigen in fetal tissues has been documented; although, the route of transmission has not been elucidated. PTV have been associated with fetal losses. Oral, aerosol and/or subcutaneous inoculation of the virus in gestating sows 30 days prior to gestation resulted in embryonic death [59,76-78].

Iagtrogenic route

Transmission through the use of contaminated syringes, artificial insemination, teeth clipping, nasogastric feeding and other common routine practices are another potential alternative of virus spread. [76].

Intracellular primary replication site

The process of which virión initiates primary replication in the host cell after endocytosis is not known. Authors have conjectured by analogy with other members of the family Picornaviridae that after the virions are engulfed by endocytosis, the acidic pH of the endosome induces the initial disassembly of the virión causing conformational changes in the virion capsid with the release of the VP4 and posterior formation of an endosomal pore. After this step, the viral genomic RNA appears to be injected into the cell's cytoplasm.

After replication in the original infected cell and posterior cellular lysis, the virus is thought to gain access to blood and lymphatic vessels and results in a viremia. The detection of viral antigens by immunohistochemistry in the myenteric nerve plexus in the small and large intestine indicates that this structure is a potential replication site of the virus[68].Replication in neurons innervating the gastrointestinal tract appear to be a strategy used by the virus to gain access to the CNS by retrograde axonal transport.

Dissemination and CNS entry.

Studies have documented a transient viremia after the initial replication in the gastrointestinal tract suggesting that infectious virions are able to cross the blood-brain barrier

and establish a productive infection. Other authors have postulated that the virus enters through affected neurons of the enteric nervous system (ENS) and peripheral nervous system (PNS) and by retrograde axonal transport of infectious virions the CNS is invaded.

The Fecal-Oral Model of Pathogenesis and Virus Distribution.

The fecal-oral model of pathogenesis postulated for *Teschovirus A* infections dictates that the presence of viral RNA in the gastrointestinal tract is indicative of the presence and contamination of PTV in swine herds. The detection of viral RNA in gastrointestinal lymph nodes and/or other lymphoid organs could indicate viral penetration beyond the *tunica mucosa*; and its presence in visceral organs might be indicative of viremia. The presence of viral RNA in CNS tissue correlates with virus penetration and invasion of the CNS.

Studies detecting the presence of viral RNA targeting the 5' UTR by RT-PCR have shown that the virus is present in varied organs. Chiu *et al.* informed that the presence of PTV in endemically infected pigs was detected in decreasing order of magnitude, from the following tissues: Intestine ($\bar{x}$ 61%); Lymphoid organs ($\bar{x}$ 59%); Visceral organs ($\bar{x}$ 37%); Caudal section of brain and C1($\bar{x}$ 17%); Rostral and medial parts of brain ($\bar{x}$ -47%). In a subsequent study carried out by the same author, involving the sampling of 29 animals between 4-8 weeks of age from farrow to finish herds the detection of PTV viral RNA yielded the following results: Inguinal lymph node (100%); Iliac lymph node (89–91%); Tonsil (66–68%); Ileum (59–60%); Spleen (38–40%); Kidney (30–31%) and Brain (22.9%). In both studies, samples were obtained from 4 to 8 weeks-old pigs showing poor growth performance, gastrointestinal and respiratory symptoms. In these studies, serotypes PTV-1, -4, -6, -7, and -11 were identified.

Clinical Signs.

The clinical signs for Teschovirus encephalomyelitis are mainly associated with neurological disturbances affecting the CNS [3].

A previous study have shown that incubation period averaged 13.5 days and 8.5 days for 21 day-old pigs and 14 days-old pigs, respectively. CNS clinical signs included but were not limited to ataxia of the rear legs, excitement, paralysis, trembling with clonic and tonic spasms, recumbency, opisthotonus, nystagmus, paddling of the rear legs, flaccid paralysis and coma. The mean temperature for the 21 day-old pigs was 102.6 F and 102.2 F for the 14 day-old pigs. According to the results of this study, it appears that 14 day-old pigs were more susceptible to infection, with a shorter incubation period, higher temperature, and shorter disease course than 21 days-old pigs. A percentage of animals will eventually recover given the proper care.[44].

The comparison between the highly pathogenic strain PTV 1-Konratice and the mild strain PTV 1- Talfan was characterized by the intranasal and oral inoculation in gnotobiotic pigs. In this experiment, all infected animals developed paraplegia regardless of the inoculation route. The authors also remarked that some of the animals regained use of their forelegs but majority remained recumbent until euthanized. The authors also introduce the idea that pneumonia associated with cases of Teschovirus encephalomyelitis could be caused by the prolonged recumbency and prostration shown by affected animals resulting in hypostatic congestion of the pulmonary parenchyma [42].

Huck *et al.* also compared the clinical course of the Konratice and Talfan strain by intracerebral inoculation of virus. Thirty-three and forty-six animals were inoculated with strains

Konratice and Talfan, respectively. A low dose (1.8 to 3.4 × 10^4 PFUs) and a high dose (8.0 × 10^4 and 1.7 × 10^7 PFUs) inoculum groups were made for each virus strain. The age of animals ranged from twenty-tree to thirty-three days old. The authors observed that the Konratice strain regularly caused severe disease characterized by sudden onset of paralysis followed by death of inoculated animals. In contrast, the Talfan strain caused a milder disease with a longer incubation period, ataxia, paresis and paralysis as main features, and clinical recovery by most animals. [67].

Experimental inoculation with pathogenic strains of PTV-1 have been found effective at inducing polioencephalomyelitis, neurological symptoms and diarrhea when the virus is administered solely by the intranasal route[79].

The pathogenicity of other strains has been also studied under experimental settings. Two serotypes previously isolated from brains from affected pigs, PTV-2 strain O3b and PTV-3 strain O2b were inoculated into germ free and pathogen free pigs. Polioencephalomyelitis and specific neurological signs including flaccid and spastic paralysis were noted in affected pigs[80].

Also in the US, animals infected with a non-characterized serotype of PTV, but homologous to PTV-1, presented clinical disease with signs such as fever, lethargy, recumbency, tremors, paddling movements, extensor rigidity, ataxia, and paralysis[81].

The main clinical sign during an outbreak of disease caused by a PTV-1 strain affecting diverse areas of Haiti, in 2009, indicated that posterior ataxia followed by paresis and/or paralysis was the most common clinical feature. These signs were evident on the second or third day of illness with an estimated morbidity of 60% and a mortality of 40% in affected animals [82].

The presence of more than one serotype in different organs of affected animals suggest that co-infection by different serotypes of PTV is possible although its clinical relevance has not been completed elucidated [69].

Enumerated in Table 1 are common clinical signs described in cases of PTV clinical disease from a selected list of scientific paper and communications[39,40,42,44,52,56,57,61,67,70,72,79,81-86].

Nonspecific clinical signs.

Other authors have described the occurrence of nonspecific clinical signs in naturally infected animals under experimental conditions. Fever, anorexia, and depression are signs commonly described in pigs inoculated using different routes or as consequence of natural infections [42,57,66,67,70,80,85,87,88].

Table 1. Common PTV clinical signs described in literature.

Clinical sign	**Reported frequency**
Ataxia	11
Paralysis (Tetra; para)	11
Paresis	11
Fever	8
Lateral recumbency	7
Diarrhea	6
Neural disorders (sic)	5
Lethargy	3
Stiffness of legs	3
Convulsions	3
Tremors	3
Nystagmus	3
Opisthotonus	3
Paddling movements	2
Excitable to sound stimuli	2
Respiratory distress	2
Vocalization	2
Coma	2
Locomotor disorders	1
Extensor rigidity	1
Bruxism	1
Anorexia	1

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Gross Pathology

Majority of authors indicate that no gross lesions were observed at the time of postmortem examination [1,5,11,41,43,69].

Histopathology

One of the first pathological studies for this disease was conducted in 1954 by Manuelidis *et al.* [72].

This paper covers the microscopic findings of 300 pigs affected by different strains of the virus inoculated experimentally by different routes as other very important aspects of the disease. The authors recognized three different stages of the disease: early, midcourse and convalescent.

In the early stage, the authors describe that a mild, focal, lymphocytic meningitis at the base of the brain and cerebellum is a common feature. This lymphocytic infiltration was described to be located around small vessels or forming independent cell nodules. In addition, the pons and medulla contained few cell nodules and infiltrations around the small vessels. Affected neurons demonstrated characteristic features such as chromatolysis of the Nissl substance and nuclear hyperchromatism. In contrast, spinal cord lesions were not commonly described during this stage. In the few cases where lesions were found in the spinal cord, the ventral horn of the cervical cord was affected; the thoracic, lumbar, or sacral cord were not affected.

During the midcourse of disease most lesions concentrated in the ventral horns and dorsal horns of the spinal cord and no significant differences were noted between the cervical, thoracic,

lumbar, and sacral regions. Lymphocytic infiltration of the Virchow-Robin spaces with few plasma cells and histiocytes was observed in areas with or without neuronal damage.

The pathological features described during the convalescent stage of disease indicates that the large damage induced by the infection was correlated with the decrease in number of neurons in the affected areas. In the cerebellum, the molecular and granular layers were diminished in size, and Purkinje cells were missing over large areas. Sections of spinal cord revealed obvious loss of neurons in the ventral horns and few lymphocytes and plasma cells were present around blood vessels in addition to the proliferation of astrocytes and microglial cells. In other regions of the CNS, perivascular infiltrations nor cell nodules were present.

Similar microscopic lesions had been described by other authors under different experimental settings using other strains.

Dardini *et al.* demonstrated that animals infected with PTV-1 Konratice developed a non-suppurative encephalomyelitis characterized by the degeneration and necrosis of neurons, particularly affecting the grey matter at the ventral horns of the spinal cord, the Purkinje cell layer of the cerebellum, the medulla oblongata, pons, midbrain, thalamus, and basal ganglia. A lymphocytic perivascular infiltration was commonly present in areas adjacent to the affected neurons. [44].

Long *et al.* described the lesions caused by PTV-2 strain O3b and PTV-3 O2b as a non-suppurative encephalomyelitis characterized by neuronal degeneration, neuronophagia and perivascular infiltration of advential cells and microglia. Lesions were commonly observed within the gray matter of the spinal cord, brainstem, cerebellum and less frequently in the cerebrum[80] [71].

Yamada *et al.* demonstrated that animals challenged with PTV-1 strain Toyama 2002 had consistent perivascular infiltration of mononuclear cells, focal areas of gliosis, neuronal necrosis and neuronophagia in the brainstem, cerebellum, and spinal cord. Severe ganglioneuritis of the spinal ganglion and neuritis in the spinal root were also described [89].

In another study by Yamada *et al.*, Teschovirus encephalomyelitis was experimentally induced in three-week-old piglets via intravascular inoculation. Similarly to other experiments, diffuse non-suppurative encephalomyelitis was observed in affected pigs. Furthermore, specific PTV antigens were detected by immunohistochemistry and positive staining was often associated with histologic lesions. Antigens were also detected in dorsal ganglion cells, spinal dorsal root, and spinal cord[70].

Epidemiology

The Wild board (*Sus scrofa*) and domestic pig (*Sus Scrofa f. domesticus*) have been identified as the exclusive natural host for *Teschovirus A*[72]. It is unknown whether other animals are susceptible to natural infection[90]. The transmission of the virus to susceptible animals is through both indirect and direct contact with infected animals and their secretions.

The virus is widely disseminated throughout the world and is ubiquitous in swine populations[2]. *Teschovirus A* has been identified in all continents except Antarctica. The presence of the virus or serological evidence has been reported in the following countries within the last century Czech Republic[91], Slovakia[92] , United Kingdom[10], Germany[93,94], Austria, Latvia, Poland[95], Romania, Belarus, Croatia, Hungary[6], Switzerland[59,96], Ukraine[97], Russia, Denmark[11], France[98], Italy[99], Spain[5], Sweden[86], Norway[92], in

Europe; Madagascar[73], Island of Reunion (France), South Africa[100], Uganda, in Africa[92]; Japan[17,87], China[101,102], Republic of China (Taiwan)[103], Republic of Korea[56], In Asia; Bolivia[64], Brazil[62], Colombia[92], Dominican Republic[104], Haiti[82], USA[16,81,105], Canada[15,85,92], in the Américas; and New Zealand[106] and Australia[28,107,108].

Surveys performed in different parts of the world indicate that the virus is widely distributed and affected herds generally show a high prevalence of infection. In Taiwan, a serological survey indicated that more than 70% of the swine population in this country were exposed to PTV-5.

Another experiment carried out in China by Zheng *et al.*, demonstrated a high seroprevalence across different strains of PTV in some northeast provinces of the country. In this experiment, it was found that 61.3% of 1384 sera samples were positive for PTV-8, although other serotypes (PTV-2, -4, -6) were also detected. This study also concluded that all sampled herds were seropositive for PTV. [102].

In Japan, 76.7% of 408 animals were serologically positive to different serotypes of PTV. In Spain, 47% of 600 porcine fecal samples surveyed were positive for PTV and 206 isolates were obtained [5,109]. A six year cross-sectional study carried out in Czechoslovakia reported a high prevalence of PTV is swine showing that co-infection with different serotypes of PTV is a common epidemiological feature[110].

A survey involving the detection of *Teschovirus A,* Sa*pelovirus A* and *Enterovirus G* in feces of domestic pigs and wild boars of Czech Republic between 2005 and 2011 documented that 69.4% of domestic pigs and 63.3% of wild boars were PCR positive to two or three different serotypes [111].

In Madagascar, Taiwan and Haiti, the virus is considered endemic [79,82].

Outbreaks of highly virulent PTV-1 strains (Teschen disease) were associated with high mortality and mobility, and occurred sporadically throughout the world, although more recently, strains previously described as low virulent strains of PTV-1 (*e.g.* Talfan strain) have acquired notoriety [84].

Other serotypes of PTV (PTV 2-13) are considered of low virulence and much of their epidemiological burden is not known.

The identification of the wild board as the natural reservoir for these viruses is commonly accepted although the transmission of the virus from domestic swine to the environment and consequently to wild boars is also an important epidemiologic characteristic to consider [112,113].

The epidemiological implications of animals of the genus *Pecari* (Peccary), *Babyrousa* (Babirusa) and *Phacochoerus* (Warthog) has not been investigated.

It has been postulated that geographic location and climatic conditions can influence the prevalence and number of clinical cases. Infections can occur year-round in latitudes where the virus can survive during the winter season and it is not inactivated by low temperatures. In cold latitudes, the outbreaks of disease are commonly described to occur during the spring and summer seasons[114].

Given its socio-economic relevance, the disease was considered of major importance for international trade and commerce of animals and animal products. Since its publication in 1959, the FAO/WHO/ OIE Animal Health Yearbook included Teschovirus encephalomyelitis in a list of internationally notifiable diseases. In 1986, after the reorganization of the OIE classification system for animal diseases, "Teschen disease" was included with 16 other animal diseases as an

"OIE List A disease" under the code number A140. The criteria for inclusion to this list was defined by the OIE as a "Transmissible disease that has the potential for very serious and rapid spread, irrespective of national borders, that are of serious socio-economic or public health consequence and that are of major importance in the international trade of animals and animal products". In 1995, the OIE reclassified and moved the disease to the "OIE List B diseases" under the code B526. In 2005, the OIE's International Committee and regional commissions instructed the OIE headquarters to establish a single OIE list of notifiable diseases, and as a result of these directives, in 2006 the disease Teschovirus encephalomyelitis was eliminated and its notification was abolished.

Prevention and Control Measures

Complete eradication of the causal agent in most of commercial herds is a virtually impossible task. Usually, control measures primarily focus on decreasing the likelihood of the introduction of a highly virulent strain rather than on the treatment of affected animals.

Disease control and spread have been successfully achieved by the implementation of non-specific strategies including stamping out of affected areas, vaccination in response to the outbreak, restriction of animal movements, official destruction of animal products, official disposal of carcasses and animal by-products, and disinfection of premises.

The use of antivirals drugs to ameliorate clinical signs has not been investigated. Supportive therapy can be administered in pigs with polioencephalomyelitis. The efficacy of non-steroidal and steroidal anti-inflammatory drugs in cases of TE is not well documented in the scientific literature.

No efforts to raise PTV free herds have been documented although the accidental introduction of the virus in specific pathogen-free herds has been described [115].

Currently, disease prevention in countries where highly pathogenic strains of PTV are not endemic is attained mostly by practicing strict biosecurity measures. Additionally, a timely and appropriate diagnosis followed by the implementation of biosecurity measures theoretically should minimize the spread and perpetuation of disease in a specific population and geographic location.

PTV-2 and PTV-11 strains

PTV 11 strains

As of March 1st 2017, the GenBank database contained four pathogenic PTV-11 nucleotide sequences under the taxonomy ID: 363171. All strains were described to occur in Europe. These are the prototype strain Dresden, which was described by Hahnefeld *et al.* in 1965[116]; Strain DS 1696/91 described by Appel *et al.* in 1991[117], and strains RD 181/01 and 1008/88 isolated in different research facilities in Germany.

Pathogenic strains

Strain Dresden

The prototype strain Dresden was first described by Hahnefeld *et al.* in 1965 and was initially catalogued as a PEV 1. The neuropathogenicity of this strain was first identified after an outbreak of disease identified in 1963 in the district of Dresden, Germany. This outbreak caused transient ataxia of varying severity and death in piglets of 4-6 weeks. The isolation of the virus was achieved by inoculating porcine kidney cells with CNS tissue homogenate. In 2001, Zell *et*

al. indicated that this strain, in addition to strains UKG 53/81 and DS 1969/91, should be recognized as a distinct serotype based upon their intrinsic antigenic and molecular similarities given the immunological reaction to specific monoclonal antibodies MAb 040/4B1 and MAb 041/3C3 [118].

Strain DS 1696/91

Strain DS 1696/91 was described by Addel *et al.* in 1995. This strain was identified and further isolated from brain homogenate samples collected from animals showing neurological symptoms compatible with Teschovirus encephalomyelitis at the Food and Veterinary Examination Office of the State of Schleswig-Holstein (Lebensmittel- und Veterinäruntersuchungsamt des Landes Schleswig-Holstein, LVUSH) Neumünster, Germany[119].

Strain RD 181/01

Strain RD 181/01 was isolated in 2001, at the Food, Medical and Veterinary Examination Office of the state of Thüringer (Thüringer Medizinal-, Lebensmittel- und Veterinäruntersuchungsamt, LVLUAMV) Bad Langensalza, Germany[119].

Strain 1008/88

Strain 1008/88 was isolated in 1988, from spinal cord of affected animals showing neurological disorders at the Food, Medical and Veterinary Examination Office of the state of Thüringer (Thüringer Medizinal-, Lebensmittel- und Veterinäruntersuchungsamt, TMLVUA) Bad Langensalza, Germany[119].

Other strains

Other nine PTV-11 strains are indexed in GenBank. The pathogenicity of these strains has not been assessed. Strain UKG 53/81 was initially isolated from feces of healthy animals at the Institute for Animal Health Pirbright Laboratory (IAHPL), England[119]. Strain Vir 2374/01 was identified in Germany from a swine host. In Italy, La Rosa *et al*. identified six more PTV-11 strains in feces from healthy animals[120]. In 2009, Buitrago *et al*. identified in Spain a strain designated CC82 from feces of healthy animals[109]. A list of PTV-11 strains identified for this thesis is available in Annex A.

PTV-2 strains.

Ten pathogenic PTV-12 strains are indexed in GenBank under the taxonomy ID: 138679. These are prototype strain T-80 (isolated in UK); Strains Vir 6711-12/83, Vir 6793/83, Vir 480/87, Vir 2018/87, DS 756/93 and DS 183/93 (isolated in Germany), and two strains isolated in Asia: the Japanese Strain SFK10 (previously known as serotype J2) and Chinese Strain JF613.

Pathogenic strains

Strain T-80

The PTV-2 prototype strain T-80 was originally isolated in 1960 by A.O. Betts *et al.* from the tonsils of healthy animals at Cambridge Veterinary School, England [121]. The neuropathogenicity of this strain was also demonstrated by Betts *et al.* when inducing CNS signs and histological lesions as polioencephalomyelitis in colostrum deprived, pathogen-free piglets. These animals were inoculated intranasally, orally or intracerebrally. The estimated incubation

period reported was between 7 to 20 days and classic clinical signs of Teschovirus encephalomyelitis were reproduced. The authors also described cellular lesions as neuronal degeneration and necrosis of CNS parenchyma, perivascular cuffing of Virchow-Robins spaces and multiple nodular areas of glial cells. Lesions were found particularly in the spinal cord and brainstem.

Strains Vir 6711-12/83, Vir 6793/83, Vir 480/87, Vir 2018/87

These four strains were originally isolated at the State Veterinary Examination Office (Staatliches Veterinäruntersuchungsamt Arnsberg, SVUA) in Arnsberg, Germany. All strains were isolated from brain samples of pigs showing CNS clinical signs compatible with Teschovirus encephalomyelitis[119].

Strains DS 756/93 and DS 183/93

Both strains were isolated at Lebensmittel un Veterinäruntersuchungsamt des Landes Schleswig-Holstein (Food and Veterinary Examination Office of the State of Schleswig-Holstein, LVUSH), Neumünster, Germany. Strains DS 756/93 was isolated from a brain sample of CNS affected pigs. Strain DS 183/93 was isolated from an unknown sample from pigs showing neurological disorders[119].

Strain Sek 49/99

Strain Sek 49/99 was isolated at the Institute for Animal Breeding, Animal Husbandry, and Animal Health (Institut für Tierzucht, Tierhaltung und Tiergesundheit, ITT), Oldenburg,

Germany. The virus was isolated from a pool of tissues (spleen, lymph nodes, lung and liver) from animals with non-specific reproductive and respiratory clinical signs[119].

Strains 2-AK-III and 12-PL

Strains 2-AK-III and 12-PL identified and described by Szent Ivanyi *et al* [122]in 1963 in Hungary.

Strain SFK10

Japanese Strain SFK10 (previously known as serotype J2) was identified by Morimoto *et al.* in 1962 after its isolation from feces [123].The experimental inoculation of this strain did not induced clinical signs in inoculated animals although the presence of neuronal degeneration, glial nodules, and perivascular infiltration of mononuclear cells in CNS histological sections were evident in one piglet after 10 days post inoculation.

Strain JF613

The Chinese Strain JF613 was identified by Wang *et al.* in 2010[124]. This strain was isolated from brain samples of pigs suffering clinical disease characterized by fever, diarrhea, respiratory distress, lateral recumbency and paralysis of the rear legs. A virulence study was conducted in 5-week-old piglets (Negative for pathogens as PTV, PRRSV, CSFV and JEV) although no results were communicated. The authors also indicated that the origin of this strain could be the result of a natural recombination event and claimed that strain JF613 has a mosaic genomic structure compatible with two previously reported PTV-2 and PTV-5 isolates.

Strain o3b

Strain o3b was identified by Kasza *et al.* in 1965[125]. The virus was isolated from brain samples of affected animals with polioencephalomyelitis. The pathogenicity of this virus was demonstrated by Long *et al.* by intracerebral inoculation and oral administration of the virus in germfree pigs, resulting in clinical disease [80]. The oral administration of the virus induced a more severe disease characterized by flaccid and spastic paralysis. A nonsuppurative encephalomyelitis was observed in histologic sections of CNS of affected animals with lesions. Neuronal degeneration, neuronophagia and perivascular cuffing were described as common histologic lesions. The authors indicate that lesions were widely distributed at the grey matter in all sections of cerebellum, brain stem and spinal cord evaluated.

Strain 6335

This U.S. strain was described by Janke *et al.* in 1988[66]. No CNS clinical disease was observed in animals orally inoculated with this strain. The inoculation of this strain in addition to a group A serotype 1 porcine rotavirus was assessed and animals developed watery diarrhea 18-24 hours post inoculation. The authors concluded that this PTV strain did not induced clinical signs nor macroscopic or histologic lesions by itself and suggested that coinfection with rotavirus A did not had any synergistic effect. On the contrary, authors indicated that strain 6335 could ameliorate the signs and lesions observed when compared to rotavirus infections alone.

Other strains

Forty-nine more PTV-2 strains are indexed in GenBank. The pathogenicity of these strains has not been assessed. Thirty-two strains from feces were identified by a survey conducted by Buitrago *et al.* in Spain [5]. La Rosa *et al.* also identified in feces fifteen PTV-2 strains in Italy[120]. Strain ZJ16LX0401 was identified in China in 2016. Strain Stendal 2532 was reported by Zell *et al.* from an unknown sample provided by the National Veterinary and Food Research Office (Landesveterinär- und Lebensmitteluntersuchungsamt, LVLUASA) in Sachsen-Anhalt, Germany[119].

Table 2. List of PTV-11 and PTV-2 pathogenic strains.

Serotype	GenBank Accession	Strain	Year of isolation	Virus isolated from	Country	Reference
PTV-11	AF296096	Dresden	1965	CNS	Germany	Hahnefeld *et al.*
	AF296121	DS 1696/91	1991	Brain	Germany	Appel *et al.*
	AY392536	RD 181/01	2001	N.A.	Germany	Zell *et al.*
	AF296120	UKG 53/81	1981	Feces	United Kingdom	Zell *et al.*
	AY392550	1008/88	1988	Spinal cord	Germany	Zell *et al.*
	GQ293238	Vir 2374/01	2001	N.A.	Germany	Zell *et al.*
PTV-2	AF296087	T-80	1960	Feces	United Kingdom	Betts *et al.*
	AF296110	Sek 49/99	1999	Organ pool	Germany	Zell *et al.*
	AF296107	Vir 6711-12/83	1983	Brain	Germany	Auerbach *et al.*
	AF296108	Vir 6793/83	1983	Brain	Germany	Auerbach *et al.*
	AF296109	Vir 480/87	1987	Brain	Germany	Auerbach *et al.*
	GQ293229	Vir 2018/87	1987	N.A.	Germany	Zell *et al.*
	AY392534	DS 756/93	1993	Brain	Germany	Zell *et al.*
	AY392533	DS 183/93	1993	N.A.	Germany	Zell *et al.*
	AY392542	2-AK-III	1963	Rectal swab	Hungary	Szent-Ivanyi *et al.*
	AY392541	12-PL	1963	Rectal swab	Hungary	Szent-Ivanyi *et al.*
	AB049554	SFK10	1965	Feces	Japan	Morimoto *et al.*
	GU446660	JF613	2010	Brain	China	Wang *et al.*

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Diagnosis

Subclinical infection is an important epidemiological feature shown by *Teschovirus A.* It is well established that PTV serotypes are ubiquitous in healthy swine populations and identified in field samples from pigs of all ages. The mere identification of the virus in feces does not necessarily indicate clinical disease.

Historically, neurological, enteric, respiratory and reproductive signs have been reported in PTV outbreaks given the broad nature of clinical signs, an accurate diagnosis should be initiated upon proper identification of the different clinical syndromes associated with this virus.

Infections caused by *Teschovirus A*, particularly those associated with PTV-1, have global relevance. Teschovirus encephalomyelitis was a List A notifiable disease to the World Organization for Animal Health (OIE) since the inception of the FAO/WHO/ OIE Animal Health Yearbook in 1959 until 2005 when it was removed from the OIE's Terrestrial Animal Health Code. However, an exclusive chapter enumerating the accepted techniques for its diagnosis as the methods to identify this pathogen is still present in the OIE's Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

One of the objectives of the OIE's Manual is to set worldwide accepted laboratory standards. This manual includes a section exclusively dedicated to Teschovirus encephalomyelitis and describes internationally regulated diagnostics techniques. The complementary diagnostic tests indicated for this disease includes histologic examination and immunohistochemistry, identification of the agent via virus isolation, indirect fluorescent antibody test (IFA), reverse-transcription polymerase chain reaction (RT-PCR) and serological tests as virus serum neutralization and enzyme-linked immunosorbent assay (ELISA).

In outbreaks of neuropathogenic strains of PTV, a presumptive diagnosis is primarily made upon the observation of suggestive clinical signs, identification of compatible histopathologic lesions and identification of the pathogen. Final corroboration can be achieved by the implementation of classical virologic techniques (virus isolation), immunological techniques (ELISA, virus serum neutralization, virus serotyping, etc.) and molecular diagnostic techniques (PCR, Next generation sequencing, etc.). An epidemiological investigation can be carried out in order to find evidence of potential previous outbreaks.

In outbreaks with neurological signs, the recognition of non-specific early clinical signs as fever, lassitude and anorexia followed by the latter characteristic clinical signs of disease as changes in the gait, ataxia, tremors, opisthotonus, nystagmus, paralysis of cranial nerves and extremities (paraplegia and quadriplegia), convulsions, coma and death are indicative signs of disease caused by neurotrophic strains of PTV.

In general, vast majority of studies report the absence of macroscopic lesions in outbreaks of neurological disease; although, non-specific findings such as pulmonary consolidation, lymphadenomegalia, hepatic congestion and fluid and gas filled intestines have been noted.

Histological lesions commonly described in PTV outbreaks are indicative of a neurotropic virus however lesions are not pathognomonic of PTV infection. Other neurotrophic viruses such as *Sapelovirus A*, *Classical swine fever virus*, *Japanese encephalitis virus*, *Porcine hemagglutinating encephalomyelitis virus*, highly virulent strains of the *Porcine reproductive and respiratory syndrome virus*, *Rabies virus* and *Suid herpesvirus 1* should be considered as differentials. Table 3 enumerates a list of differential diagnosis of disease.

Virus isolation from CNS tissues and subsequent serotyping of the virus are acceptable diagnostic methods to corroborate PTV infections. The finding of viral antigens in CNS by *in situ* immunocytochemistry allows the diagnostician to corroborate the diagnosis. The detection of PTV nucleic acids by PCR in CNS samples obtained from affected animals is also an acceptable diagnostic method to corroborate the diagnosis of PTV, although the results from this technique relies on the proper interpretation of results by the diagnostician.

Cross-contamination of specimens during necropsy or shipment of samples is a factor to be considered at the time of interpretation.

Table 4 enumerates different diagnostic techniques used as aid in the diagnosis of neurotropic infections caused by PTV.

Table 3. Differential diagnosis of Teschovirus encephalomyelitis.

Viral agents	Disease	Agent
	Pseudorabies (Aujeszky disease)	*Suid herpesvirus 1 (Herpesviridae)*
	Classical swine fever	*Classical swine fever virus (Flaviviridae)*
	Sapelovirus A	*Sapelovirus A (Picornaviridae)*
	Japanese encephalitis	*Japanese encephalitis virus (Flaviviridae)*
	Porcine Hemagglutinating encephalomyelitis	*Porcine hemagglutinating encephalomyelitis virus (Coronaviridae)*
	Rabies	*Rabies lyssavirus (Rhabdoviridae)*
	Porcine reproductive and respiratory syndrome	*Highly virulent strains of the porcine reproductive and respiratory syndrome virus (Arteriviridae)*
	PCV2-Associated Neuropathy	*Porcine circovirus type 2 (Circoviridae)*
Bacterial agents	Edema disease	*Enterotoxigenic Escherichia coli (ETEC)*
	Bacterial meningoencephalitis cause by *Streptococcus suis*	*Streptococcus suis*
Toxic agents	Salt intoxication	
	Lead poisoning	
	Insecticide poisoning	
	High levels of Aflatoxins (B1, B2, G1, G2)	
	Ergotamine	
Other	Hypoglycemia	
	Water deprivation	

Table 4. List of diagnostic techniques reported for the corroboration of neuropathogenic *Teschovirus A* infections.

Diagnostic Technique	**Reference**
Necropsy	Long *et al.*
Histological examination	Manuelidis *et al.*
Immunohistochemistry	Yamada *et al.*
In situ immunohibridization	Yamada *et al.*
Fluorescence *in situ* hybridization	OIE manual
Gel based Polymerase chain reaction	Palmquist *et al.*
Nested Polymerase chain reaction	Zell *et al.*
Real time Polymerase chain reaction	Cano *et al.*
RT- LAMP	Wang *et al.*
Indirect fluorescent antibody test in cell culture	OIE Manual
Serum virus neutralization	Dunne *et al.*
Virus isolation	Trefny *et al.*
Enzyme-linked immunosorbent assay	OIE Manual
Next generation sequencing	Matias Ferreyra *et al.*

CHAPTER 3. CASES OF TESCHOVIRUS ENCEPHALOMYELITIS IDENTIFIED A ISU-VDL DURING THE YEAR 2013-2016.

Objective

To assess the magnitude of cases of *Teschovirus A* affecting swine operations in North America, a focused search of the Iowa State University Laboratory Information Management System (ISU LIMS) was conducted to identify cases with a compatible diagnosis of Teschovirus encephalomyelitis.

The first criterion required ISU-VDL submissions to test positive using a nested, gel based PTV PCR. This nested PCR technique also identifies other porcine picornaviruses including *Sapelovirus A* and *Enterovirus G*. The assay was developed by Zell *et al*. and detects PTV serotypes 1-11[126].

The second criterion used to identify *Teschovirus A* required submissions to demonstrate compatible histopathological lesions in the brain or spinal cord based on the assessment of a veterinary pathologist.

This search was limited from 2013 to 2016, since 2013 was the first year the nested PCR assay used at the ISU-VDL.

Results

Thirty-two cases were identified that fulfilled the two requirements described previously. Thirty-one were submissions from US swine and included sixteen cases in Iowa, five in Illinois,

two cases in Nebraska, Indiana and Arkansas; and one case in Minnesota, Virginia, Ohio and Texas. Only one case was submitted from Quebec, Canada.

During 2013, seventeen cases of PTV were identified to fulfil the criteria. Respectively, five, two and eight cases were identified during 2014, 2015 and 2016 (Figure 1).

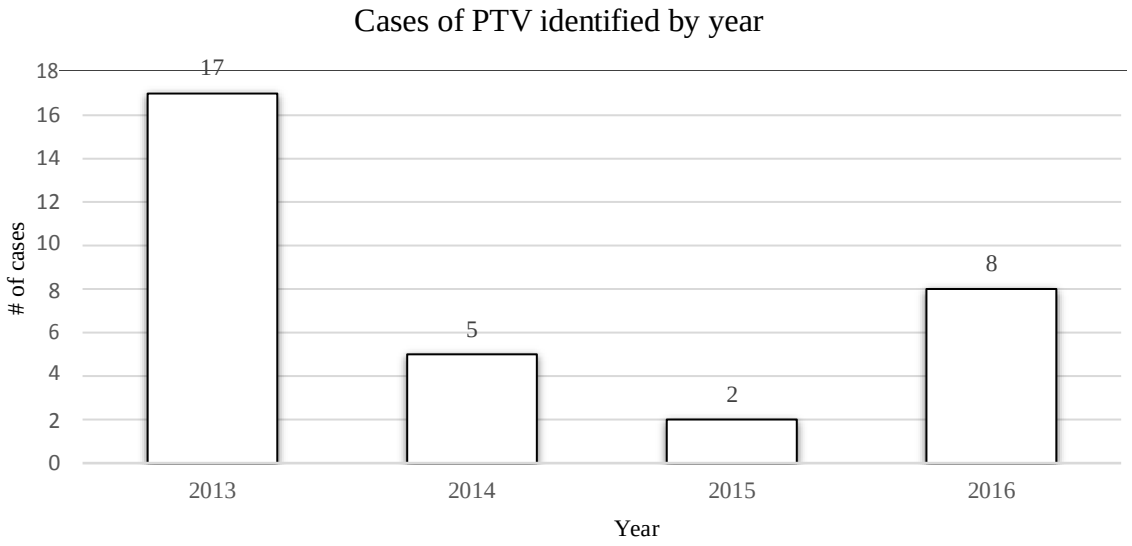

Figure 1. Cases of PTV identified by year at the ISU VDL

Conclusion

This search was merely conceived to illustrate background information obtained from cases summited to ISU-VDL from 2013 and 2016 and diagnosed as TE. No inference can be made upon the serotype given the nested PCR assay does not discriminate between the different serotypes. In addition, the frequency and distribution of cases does not represent the actual epidemiological prevalence of PTV cases occurring in North America and represents cases diagnosed as PTV-infection at ISU-VDL.

CHAPTER 4. POLIOENCEPHALOMYELITIS IN CESAREAN-DERIVED COLOSTRUM-DEPRIVED PIGS FOLLOWING EXPERIMENTAL INOCULATION WITH *TESCHOVIRUS A* SEROTYPE 2 OR SEROTYPE 11.

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Abstract: Teschovirus encephalomyelitis is a sporadic disease associated with *Teschovirus A* (PTV) serotype 1 and, less frequently, other serotypes. In recent years, the number of cases submitted to the Iowa State University Veterinary Diagnostic Laboratory with a history of posterior paresis has increased. Submission histories from various regions of the United States suggest a trend for clinical disease to persist in herds and affect a wider age-range of pigs than historically reported. Polioencephalitis and/or myelitis was consistently present and PTV was detected in affected neural tissue by PCR in a portion of cases. Sequencing from two clinical cases identified PTV-2 and PTV-11. To assess neuropathogenicity of these isolates, 5-week-old cesarean derived and colostrum deprived pigs were assigned to three groups: negative control (n=4), PTV-2-inoculated (n=7), and PTV-11-inoculated (n=7). Three PTV-2-inoculated pigs developed mild incoordination of

the hind limbs, one of which progressed to posterior ataxia. While all PTV-11-inoculated pigs showed severe neurological signs consistent with Teschovirus encephalomyelitis. Neurological clinical signs were not observed in sham-inoculated animals. All PTV-2- and PTV-11-inoculated pigs had microscopic lesions consistent with Teschovirus encephalomyelitis. To the author's knowledge, this is the first description of experimental PTV-11 infection demonstrating the neuropathogenicity of PTV-11 in swine in United States.

Keywords: Teschovirus encephalomyelitis; *Teschovirus A*; Porcine teschovirus; Porcine teschovirus 2; Porcine teschovirus 11; PTV; Poliomyelitis; Teschen disease; Talfan disease.

1. Introduction

Teschovirus encephalomyelitis (TE), previously Teschen or Talfan disease, is a neurologic condition of pigs commonly characterized by locomotor disturbances including ataxia, paresis, and/or paralysis [3]. Teschen disease (also known as Klobouk's disease) was first recognized in the Czech Republic in 1929 as a fatal encephalomyelitis of pigs caused by a highly pathogenic strain of *Teschovirus A* (formerly named *Porcine enterovirus* and then *Porcine teschovirus*)[91]. *Teschovirus A* consists of 13 (sero)types, *porcine teschovirus* (PTV) 1 to 13, and is a single-stranded, linear, non-segmented RNA virus of the genus *Teschovirus*, family *Picornaviridae*[1]. To date, outbreaks of mild disease have been described in United States (US) [17,52,81,84], although the most severe form of TE

caused by highly virulent strains of PTV-1 has not been reported. However, outbreaks of TE due to PTV-1 have been recently reported in Haiti, Dominican Republic, and Canada[82,85,104].

Historically, PTVs were classified within the genus *Enterovirus* based on viral morphology, cytopathic effect (CPE), serological assays and replication in different cell lines [17,25,26]. Recently, PTVs have been reclassified by nucleotide sequence matching, genomic organization, and phylogenetic analysis[126-128]. *Porcine enterovirus* (PEV)-1 to -7 have been renamed PTV-1 to -7 and PEV-11 to -13 were renamed PTV-8 to -10. PTV-11 to -13 are the (sero)types most recently identified[109,113,118]. Despite the reported high prevalence of PTV in feces, clinical disease is observed sporadically[5,6,129,130]. Although multiple serotypes have been identified and genetically characterized, the neuropathogenicity of some of these serotypes has not been fully elucidated. The aim of this study was to 1) develop an experimental model of TE and 2) determine the neuropathogenicity of the PTV-2 and PTV-11 isolates.

2. Materials and Methods

Virus inoculum. Strains PTV-2 USA/IA65463/2014 and PTV-11 USA/IA09592/2013 were isolated at the Iowa State University Veterinary Diagnostic Lab (ISU VDL) from samples of central nervous system (CNS) tissue from pigs with neurologic disease. These viruses were first identified using a previously described nested polymerase chain reaction (PCR)[131] and further characterized by virus isolation and molecular

sequencing of the VP1 capsid protein. For experimental inoculation, PTV-2 passage 8 and PTV-11 passage 7 were grown in porcine kidney 15 (PK-15; ATCC CCL-33) cell line. Nucleotide sequence data for both isolates is available in GenBank under accession numbers KY594021 (PTV-2 USA/IA65463/2014) and KY594022 (PTV-11 USA/IA09592/2013).

Next generation sequencing and phylogenic analysis. Complete genome sequences of PTV-2 USA/IA65463/2014 and PTV-11 USA/IA09592/2013 isolates were determined by next generation sequencing on MiSeq platform (Illumina, San Diego, CA) following previously established procedures[132]. Total DNA/RNA was extracted from virus cell culture and purified with MagMAX viral RNA isolation kit (Life Technologies, Carlsbad, CA) and a Kingfisher 96 instrument (Thermo Scientific, Waltham, MA)[133]. DNA was then removed with RNase-Free DNase Set (Qiagen, Valencia, CA) from the total DNA/RNA, and the remaining RNA was purified with Agencourt® RNAClean® XP (Beckman Coulter, Indianapolis, IN) kit according to the manufacturer's directions. Purified RNAs were fragmented to approximately 250bp and reverse transcribed to double stranded DNAs with NEXTflex™ Rapid RNA-Seq Kit. DNA library was constructed with Nextera XT DNA library preparation kit (Illumina, San Diego, CA). Sequencing was performed on MiSeq with 300-cycle MiSeq Reagent Micro Kit v2 (Illumina, San Diego, CA) to generate paired-end 2 x 150 bp reads for each sample. PTV sequences were extracted from raw sequencing output with BWA-MEM (v 0.7.15)[134] as previously

described with modification[135]. Specifically, reference genome library in the analysis pipeline was built by searching against NCBI nucleotide database with query '(complete genome) AND "Teschovirus" [porgn:_txid118139]'. Extracted PTV fragments were assembled with ABySS (v 1.5.2)[136] and SeqMan Pro version 11.2.1 (DNASTAR, Inc., Madison, Wisconsin) as previously described[133]. For phylogenic analysis, both PTV-2 USA/IA65463/2014 and PTV-11 USA/IA09592/13 were aligned with 79 PTV polyprotein sequences obtained from GenBank. Multiple sequence alignment and sequence comparisons were carried out using the ClustalW algorithm by Geneious® R9 software. Phylogenetic trees were reconstructed with the Neighbor Joining (NJ) method using the Tamura-Nei model by Geneious® R9 software. The confidence of the internal branches was evaluated performing 100 bootstrap replications.

Animals. Eighteen cesarean-derived colostrum-deprived pigs (CDCD) were purchased from a commercial source. Fecal swabs were collected 5 days prior to inoculation and tested using a nested PCR targeting PTV, *Sapelovirus A* and *Enterovirus G* [20]. All samples were negative. All procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University (Log Number: 6-15-8040-S).

Experimental design and clinical evaluation. Pigs were randomly assigned to three treatment groups: negative control (n=4); PTV-2-inoculated (n=7); and PTV-11-inoculated (n=7). Each group was housed in separate rooms and fed ad libitum with commercial feed.

Pigs were sedated with intramuscular injection of Telazol 500mg, Ketamine 250mg, Xylazine 250mg at 4.4mg/kg prior to inoculation. The inoculum was administrated intravenously using a 25-gauge butterfly catheter (Terumo™ Surflo™, Terumo Corporation; Shibuya, Tokyo, Japan) ensuring the delivery of virus. Negative control animals were inoculated with 3 ml of Eagle's minimum essential medium (MEM); animals in PTV-2- and PTV-11-inoculated groups were inoculated with 3 ml of 10^6 TCID50/ml of PTV-2 USA/IA65463/2014 or PTV-11 USA/IA09592/2013 (Table 1). Animals were evaluated every 48 hs for the presence of clinical signs (Table 2). Serum samples were collected on day post-inoculation (DPI) 0, 11 and 21.

Table 1. Experimental design.

Groups	Inoculum	Passage	# of animals	Route of inoculation
Control	Cell culture media[1]	-	4	Intravenous
PTV-2	10^6 $TCID_{50}$ PTV-2	8	7	Intravenous
PTV-11	10^6 $TCID_{50}$ PTV-11	7	7	Intravenous

[1] Eagle's minimum essential media.

Table 2. Mentation and ambulation scoring system.

Score	Mentation	Ambulation Score
0	Normal	Normal
1	Reduction in alertness	Mild incoordination of rear legs
2	Marked depression and head tilt	Intermittent ataxia of rear legs
3	2 plus seizures or opisthotonus	Anterior and/or posterior ataxia +/- knuckling
4	3 plus events of seizures and opisthotonus	Posterior paresis, paralysis or quadriparesis

Necropsy. Eight pigs were necropsied at DPI 11 (negative control: n=2; PTV-2-inoculated: n=2; PTV-11-inoculated: n=4). At DPI 16, a PTV-2-inoculated pig was necropsied due to clinical signs. The remaining piglets were necropsied at DPI 21. Prior to euthanasia a video of each animal was taken. A set of tissues including cerebrum; cerebellum; brainstem; spinal ganglion; sciatic nerve; and cervical, thoracic, and lumbar sections of spinal cord were fixed in 10% formalin for histopathologic examination.

Histopathologic examination. All nervous system tissues were processed for routine histopathologic evaluation. Briefly, after 48 hr fixation on 10% buffered neutral formalin, tissue were embedded in paraffin blocks, cut on 4 μm sections, and stained with hematoxylin and eosin. All sections were scored by two veterinary pathologists (PA and BA) double blinded to the study. Lesions were scored based on foci of gliosis and cellular infiltration of Virchow-Robins spaces (Table 3).

Table 3. Histopathologic severity score.

Score	# of cell layers in Virchow-Robin spaces	Areas of gliosis
0	None	None
1	1	1-2 foci
2	2	3-4 foci
3	3	> 4 foci
4	>3	-

Serum neutralization test. Antibody neutralizing activity was evaluated at 0, 11 and 21 DPI. Results were corroborated by performing an indirect immunofluorescence assay (IFA). Briefly, all sera samples were inactivated at 56°C for 30 min and aliquots of 75 µl were two-fold diluted in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum, 100 I.U./ml penicillin, 100 µg/ml streptomycin, 50 µg/ ml gentamicin and 0.25 µg/ml amphotericin B. An equal volume of diluted serum and PTV-2 or PTV-11 at 150 $TCID_{50}$/75 µl was incubated for 1 hr at 37°C in a 5% CO2 humidified atmosphere. Afterward, 100 µl of the serum-virus mixture was added to microplate wells containing pre-confluent PK-15 monolayers and incubated at 37°C. Cell controls were set up by adding 100 µl of DMEM to monolayers. Virus controls were made adding residual incubated virus stock to monolayers. After three days, monolayers were assessed daily for the presence of CPE. On the fifth day, cells were fixed with a solution of 80% ethanol at 4°C and infected cells were incubated with 50 µl of Porcine teschovirus/enterovirus antiserum (reagent code: 362-PDV, National Veterinary Service Laboratory, Ames, IA, USA) for 1 hr at 37°C, followed by 3 washes with PBS-T. Cells were incubated with 50 µl of fluorescein isothiocyanate (FITC) labeled anti-swine IgG (gamma) (KPL Inc. Gaithersburg, Maryland, USA) and incubated for 1 hr at 37°C. The presence of positive infected cells was confirmed by fluorescence microscopy (OLYMPUS IX71; Olympus Corporation, Tokyo, Japan). The reciprocal of the highest serum dilution resulting in >90% reduction of staining as compared to the negative serum control was defined as the VN titer of the serum. A VN titer of ≥8 was considered positive.

Real-time reverse transcription polymerase chain reaction. For the extraction of nucleic acids from CNS tissues, pooled sections of brainstem, cerebellum, cerebrum, and spinal cord from each animal were homogenized with Earl's minimum media. Feces were collected at the time of necropsy and immersed in 1ml of PBS. Both samples were stored at -80°C until further processing. Briefly, nucleic acids were extracted from aliquots of 50 µl of sample using MagMAX™-96 Total RNA Isolation Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and a KingFisher™ Flex Purification System (Thermo Fisher Scientific) following the instructions of the manufacturer. Primer and probe combinations for 5' Taq nuclease assay using fluorescent 3' minor groove binding DNA probe were designed targeting the 5' UTR. Primer and probe sequences are: PTV-forward GGTGGCGACAGRGTACAGA, PTV-reverse CCTGCATTCCCRTACAGGAACT and PTV-probe FAM- TGCRTTGCATATCCCTAG-MGB-BHQ. Amplification was carried out with a commercial RT-PCR kit (QuantiTect Virus + ROX Vial Kit, Qiagen), according to the manufacturer's instructions. The final protocol consisted of the addition of 5 µl of isolated RNA to 20 µl of RT-PCR mix (5 µl of 5x QuantiTect Virus Master Mix, 0.25 µl of QuantiTect Virus RT Mix, 1.0 µl XENO LIZ internal control reagent, Life Technologies, plus primers, to a final concentration of 0.4 µm; fluorogenic TaqMan MGB probe, to a final concentration of 0.2 µm; and RNase-free water up to 20 µl), and then the tubes were subjected to a first RT step at 50°C for 20 min, followed by 5 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Virus re-isolation. For all animals, aliquots of CNS tissue homogenate used for PCR were centrifuged at 800 *g* for 10 minutes at 4°C and supernatants were filtered using 0.22 µm filters (Millex, Millipore). A 100 µl aliquot was inoculated onto individual wells containing pre-confluent PK-15 monolayers and incubated for 1 hr at 37°C. After this step, the initial aliquots were discarded and culture plates were replenished with 100 µl of DMEM and incubated at 37 °C for 5 days and were examined daily for CPE. On the fifth day after a freeze-thaw cycle, the media from each well was collected separately and divided into two fractions of 50 µl each. From these, one fraction was used to re-inoculate culture plates containing pre-confluent PK-15 monolayers and the other fraction was subjected to PCR. The reinoculated culture plates were incubated for 5 days and CPE was examined daily. After this, culture plates were subjected to immunofluorescence staining to corroborate the re-isolation as described in the serology section.

3. Results

Next generation sequencing and phylogenic analysis. The genomic sequences of PTV-2 USA/IA65463/2014 isolate and PTV-11 USA/IA09592/2013 were determined using next generation sequencing technology. Phylogenetic analysis based on the polyprotein nucleotide sequence of PTV-2 and PTV-11 isolates identified in this study together with other PTV- 1 to -13 sequences were conducted. Based on the VP1 nucleotide sequence PTV-2 USA/IA65463/2014 clustered with other PTV-2 isolates and the PTV-11

USA/IA09592/2013 clustered with other PTV-11 isolates. Pairwise comparison of the nucleotide sequence of the polyprotein revealed that the PTV-2 USA/IA65463/2014 had 82.5%-89.25% nucleotide identity with 12 other PTV-2 isolates while the PTV-11 USA/IA09592/2013 had 85.8%-87.2% nucleotide identity with 4 other PTV-11 isolates (Fig 1A). Additional phylogenic analysis of the VP1 and 2/3ABC3D coding regions also corroborated that these two isolates cluster with other members of the PTV-2 and PTV-11 serotypes, respectively (Figure 1B, C).

A Polyprotein

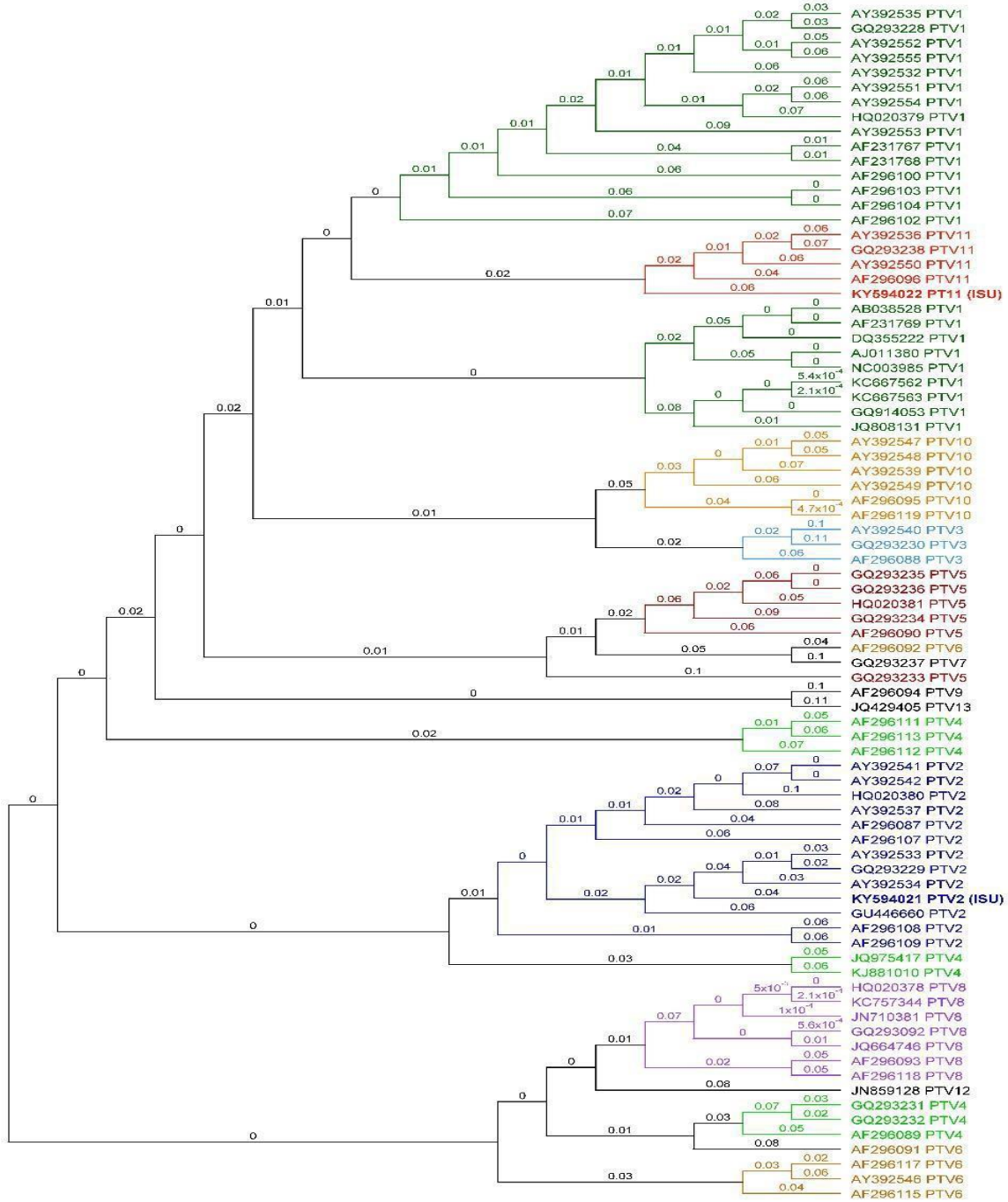

Figure 1A. Phylogenic trees illustrating the genetic relation of 79 unique polyprotein sequences with isolates USA/ IA65463/2014 PTV-2 and USA/IA09592/2013 PTV-11. Polyprotein phylogenic tree (A). Scale represents nucleotide substitutions per site.

B VP1

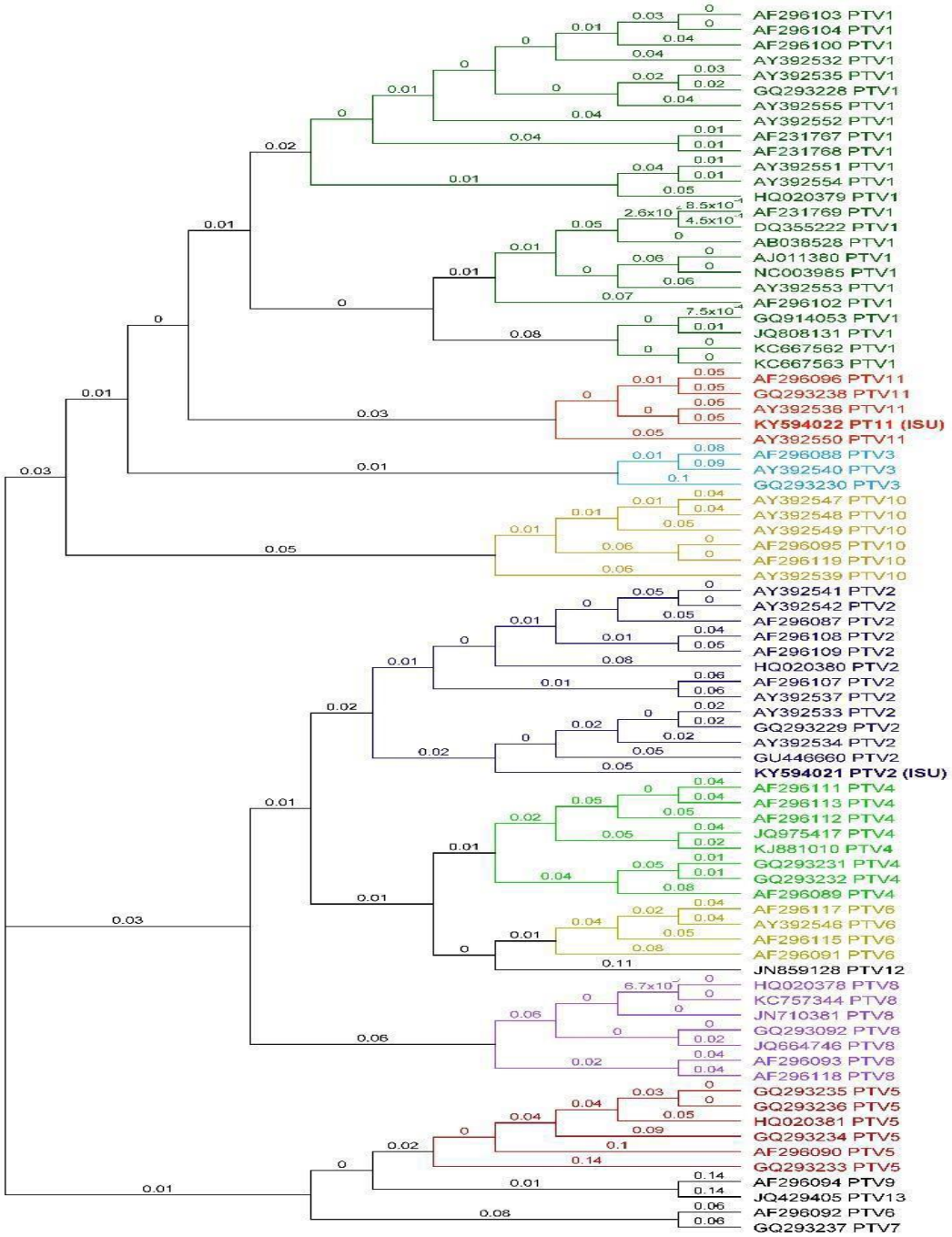

Figure 1B. Phylogenic trees illustrating the genetic relation of 79 unique polyprotein sequences with isolates USA/ IA65463/2014 PTV-2 and USA/IA09592/2013 PTV-11. VP1 gene phylogenic tree (B) Scale represents nucleotide substitutions per site.

C 2/3ABC3D

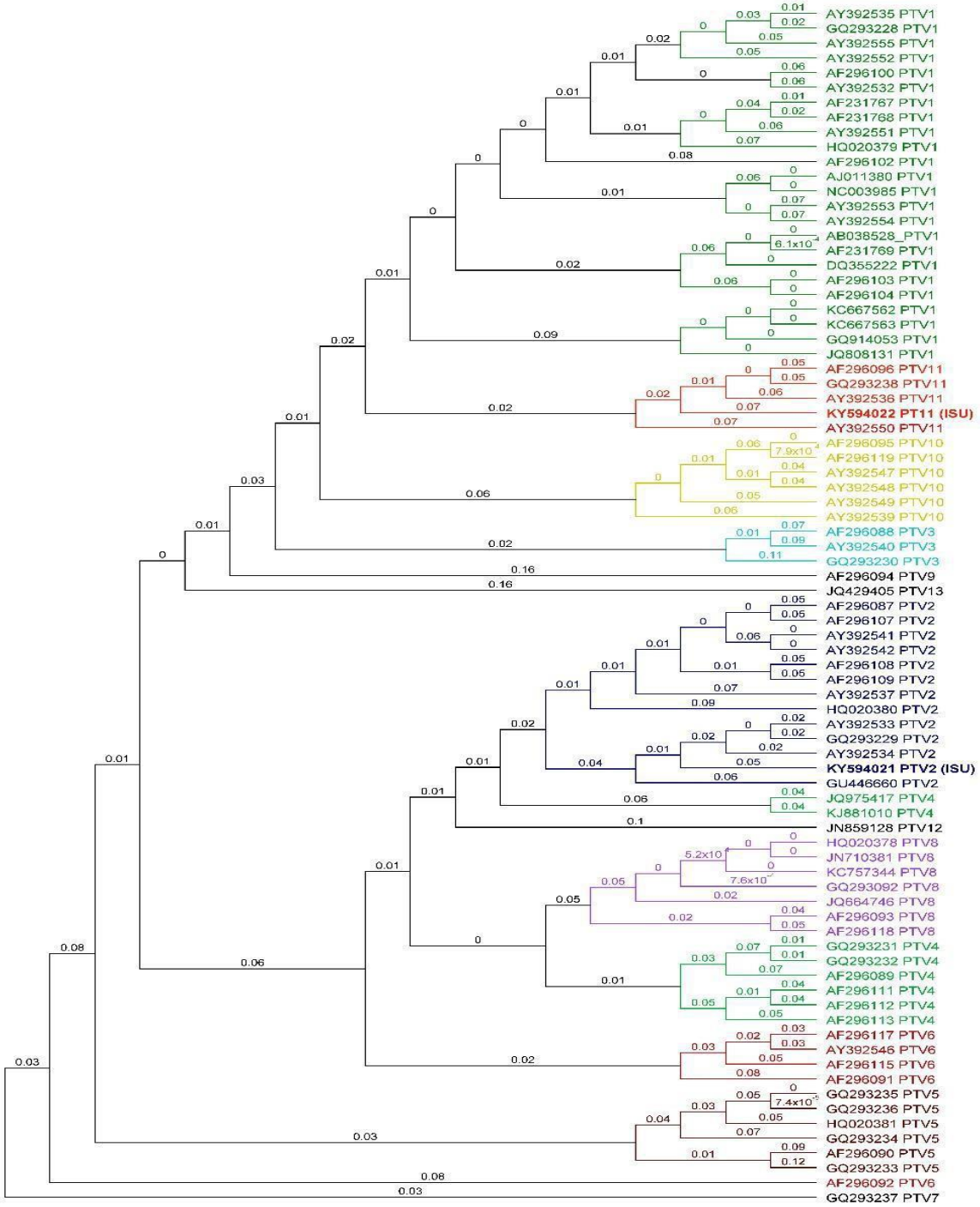

Figure 1C. Phylogenic trees illustrating the genetic relation of 79 unique polyprotein sequences with isolates USA/ IA65463/2014 PTV-2 and USA/IA09592/20 coding region2/3ABC3D phylogenic tree (C). Scale represents nucleotide substitutions per site.

Clinical evaluation. No clinical signs were observed in control animals, and all pigs remained cognitively aware throughout the study. A single pig in the PTV-2-inoculated group developed posterior ataxia at DPI 13 (Supplemental video 1). Three animals in the PTV-2-inoculated group developed mild incoordination of hind limbs starting at 15 DPI and partially recovered 4 days later. All animals in the PTV-11-inoculated group developed signs consistent with TE including hind limb incoordination, ataxia, posterior paresis and quadriparesis (Supplemental video 2) starting at DPI 9. Mentation and ambulation scoring results are summarized in Table 4. A majority of virus inoculated pigs (PTV-2: n= 5 and PTV-11: n=7) developed a nonspecific mild diarrhea beginning at DPI 7 (PTV-2) and DPI 8 (PTV-11)

Necropsy and histopathologic examination. Macroscopic examination of control and infected groups was unremarkable. All animals in the PTV-2-inoculated and PTV-11-inoculated groups presented histological lesions in the spinal cord ganglia; cervical spinal cord, thoracic spinal cord, lumbar spinal cord; obex; pons; and midbrain (Figure 2 and Table 5). The severity of the histological lesions at different levels of the spinal cord were commonly higher in the PTV-11-inoculated group compared to PTV-2-inoculated group. Lesions were noted less commonly in the cerebellum, cerebrum at the level of the diencephalon and frontal cortex in all virus inoculated pigs (Figure 2 and supplemental Figure 1).

Table 4. Clinical evaluation by animal

Serotype	Animal ID	Mentation score	Ambulation score[1]	Ambulation score by DPI[2]											Diarrhea
				1	3	5	7	9	11	13	15	17	19	21	
	61	**0**	**2**	0	0	0	0	0	0	0	1	2	2	0*	+
	67	**0**	**0**	0	0	0	0	0	0*	-	-	-	-	-	-
	68	**0**	**2**	0	0	0	0	0	0	0	1	2	1	0*	-
PTV-2	72	**0**	**3**	0	0	0	0	0	0	2	3*	-	-	-	+
	73	**0**	**2**	0	0	0	0	0	0	0	2	2	0	0*	+
	74	**0**	**0**	0	0	0	0	0	0*	-	-	-	-	-	+
	76	**0**	**0**	0	0	0	0	0	0	0	0	0	0	0*	+
	64	**0**	**2**	0	0	0	0	0	2	2	2	2	2	2*	+
	65	**0**	**3**	0	0	0	0	0	3*	-	-	-	-	-	+
	70	**0**	**4**	0	0	0	0	2	4*	-	-	-	-	-	+
PTV-11	71	**0**	**2**	0	0	0	0	2	0	0	0	0	0	0*	+
	75	**0**	**4**	0	0	0	0	2	4*	-	-	-	-	-	+
	77	**0**	**4**	0	0	0	0	2	4*	-	-	-	-	-	+
	78	**0**	**3**	0	0	0	0	0	3	3	3	3	2	2*	+

[1]Highest ambulation score noted. [2] Recorded ambulation score by day post inoculation. *Last registered score for that animal.

Table 5. Histopathologic severity score and distribution of lesions.

	Severity score[1]			Distribution	
Location	**Control**	**PTV-2**	**PTV-11**	**PTV-2**	**PTV-11**
Spinal ganglia	0	3	3	100%	100%
Cervical spinal cord	0	4	5	100%	100%
Thoracic spinal cord	0	3	5	100%	100%
Lumbar spinal cord	0	5	6	100%	100%
Sciatic nerve	0	0	0	0%	0%
Obex	0	4	6	100%	100%
Pons	0	4	5	100%	100%
Midbrain	0	4	5	100%	100%
Cerebrum plus diencephalon	0	2	4	86%	71%
Cerebellum	0	1	3	57%	71%
Front cortex	0	1	2	57%	71%

Table 6. PCR and SVN results.

Necropsy DPI	PCR (CT)[1]		SVN DPI[2]		
	Feces	CNS	0	11	21
21	0	0	1/2	1/2	1/2
21	0	0	1/2	1/2	1/2
11	0	0	1/2	1/2	-
11	0	0	1/2	1/2	-
21	26.16	33.2	1/2	1/16	1/32
11	18.23	27.8	1/2	1/16	-
21	26.24	31.6	1/2	1/32	1/16
16	18.72	30.5	1/2	1/16	-
21	22.62	32.4	1/2	1/16	1/32
11	18.79	26.9	1/2	1/16	-
21	24.78	28.6	1/2	1/16	1/16
21	21.75	31.9	1/2	1/64	1/64
11	18.59	26.2	1/2	1/64	-
11	17.78	24.4	1/2	1/32	-
21	21.64	31.3	1/2	1/32	1/64
11	18.57	24.6	1/2	1/128	-
11	20.0	24.4	1/2	1/32	-
21	21.92	32	1/2	1/16	1/32

[1] Lowest cycle threshold value in CNS samples and feces collected at necropsy. [2] Serum virus neutralization assay at day post inoculation. [3]Eagle's minimum essential media.

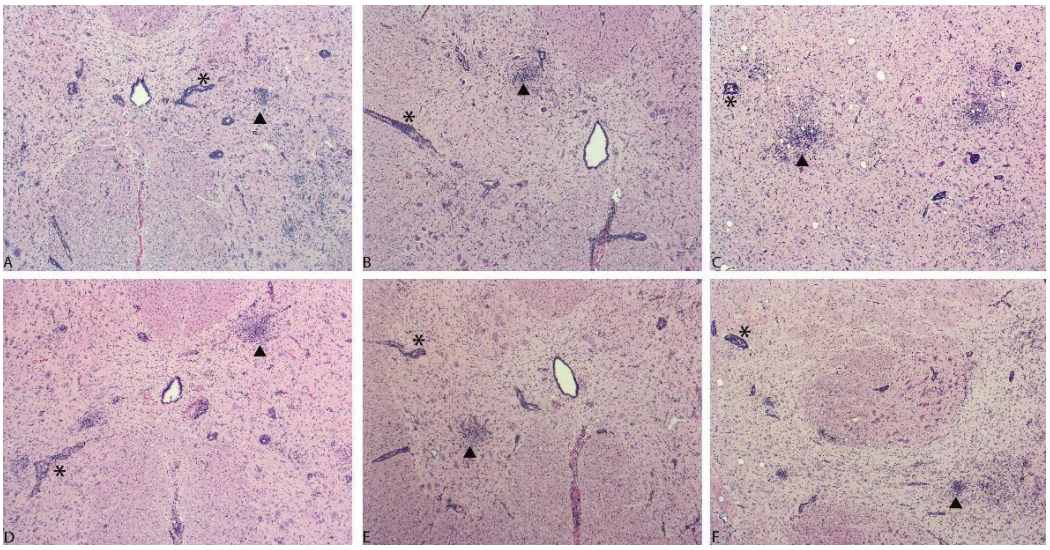

Figure 2. Pig #74, inoculated with PTV-2 (DPI 11). Lymphoplasmacytic myelitis with expansion of the Virchow-Robin spaces (*) and multifocal areas of gliosis (▲) in cervical spinal cord (A), lumbar spinal cord (B), and midbrain (C); Pig #77 inoculated with PTV-11 (DPI 11) Lymphoplasmacytic myelitis with expansion of the Virchow-Robin spaces (*) and multifocal areas of gliosis (▲) in cervical spinal cord (D), lumbar spinal cord (E), and midbrain (F).

Serum neutralization test. Serology results are summarized in Table 6. No antibodies against PTV-2 and PTV-11 were detected at DPI 0. Neutralizing antibodies were detected at DPI 11 in all virus inoculated animals and remained detectable at 21 DPI.

Polymerase chain reaction. PCR results are summarized in Table 6. PTV RNA was not detected in feces or CNS tissue in negative control animals. PTV RNA was detected in feces and CNS tissue in all infected animals in each treatment group at time of necropsy.

Virus re-isolation. CPE and positive IFA results were detected in all samples from virus inoculated animals. PTV RNA was detected by PCR in all cell culture supernatant samples. Results are summarized in Table 7.

Table 7. Results of virus isolation attempts on CNS tissues from pigs inoculated with PTV-2 or PTV-11.

Inoculum	Animal ID	CPE P0	CPE P1	IFA P1	PCR P1[1]
	61	+	+	+	33.87
	67	+	+	+	18.36
	68	+	+	+	35.13
PTV-2	72	+	+	+	31.52
	73	+	+	+	33.13
	74	+	+	+	22.11
	76	+	+	+	31.85
	64	+	+	+	34.2
	65	+	+	+	28.78
	70	+	+	+	28.42
PTV-11	71	+	+	+	21.57
	75	+	+	+	28.03
	77	+	+	+	28.14
	78	+	+	+	34.08

[1] Lowest CT detected for that animal. CPE= Cytopathic effect. IFA= Indirect immunofluorescence. P0=Passage 0. P1= Passage 1.

4. Discussion

A porcine TE model was developed that successfully demonstrated the neuropathogenicity of PTV-2 and PTV-11 serotypes. A majority of pigs in the virus inoculated groups developed mild hind limb incoordination to quadriparesis. The most severe clinical signs were noted in PTV-11-inoculated animals; however, all pigs in virus-inoculated groups developed histologic lesions consistent with TE.

Next generation sequencing of isolates USA/IA65463/2014 and USA/IA09592/2013 and subsequent phylogenetic and comparative sequence analyses confirmed these two field isolates as PTV-2 and PTV-11, respectively. USA/IA65463/2014 PTV-2 is genetically closely related to 3 European strains (AY392534, GQ293229, AY392533) isolated from CNS tissues of

swine with CNS disorders, and to a Chinese strain (GU446660) isolated from CNS tissues of swine with enteric, respiratory and CNS signs. USA/IA09592/2013 PTV-11 is genetically related to PTV-11 prototype strain (AF296096)[118] and three other PTV-11 strains (AY392550, GQ293238, AY392536) isolated in Europe. Each strain was isolated from CNS tissue of pigs with CNS disorders, with the exception of GQ293238 whose background information was not available[119]. The inconsistencies shown in the phylogenic trees for VP1 and 2/3ABC3D nucleotide genes when compared to the polyprotein gene tree agrees with previous report by Villanova *et al* [137].

Although this study was not conceived to compare the pathogenicity of these strains, less severe clinical signs were observed in the PTV-2-inoculated animals than in PTV-11-inoculated animals. Intermittent ataxia was observed in three animals and ataxia was noted in one animal inoculated with PTV-2. All animals in the PTV-11-inoculated group developed clinical signs consistent with TE with three animals developed quadriparesis. Based on the number of animals that developed clinical signs consistent with TE, the severity of clinical signs and histologic lesions, it appears that USA/IA65463/2014 PTV-2 is less virulent than USA/IA09592/2013 PTV-11. However, the authors recognize that the small sample size used in this study and inoculation route limits the interpretation of the findings and/or extrapolation to field situations. It is interesting to note that despite the mild clinical signs noted in the PTV-2-inoculated group, histologic lesions were observed in all animals.

To our knowledge, this is the first experimental inoculation using a PTV-2 autochthonous U.S. strain after the initial description of PTV-2 strain O3b made by Long *at al.* in 1966[80]. The distribution and severity of lesions are similar in both studies. Lesions are widely distributed in the CNS including spinal cord, brain stem, and cerebellum and to a lesser extent the cerebrum.

This is the first description of PTV-11 in U. S. Originally serotype PTV-11 was identified by Hahnefeld *et al.* as a PEV-1 strain in 1965[116], and was further characterized as PTV-11 by Zell *el al.* in 2001[118]. The comparative neuropathogenicity of European PTV-11 strains and this novel PTV-11 identified in the United States is not known.

Further work is warranted to assess the prevalence and geographic distribution PTV serotypes in U.S.

Supplementary Materials:

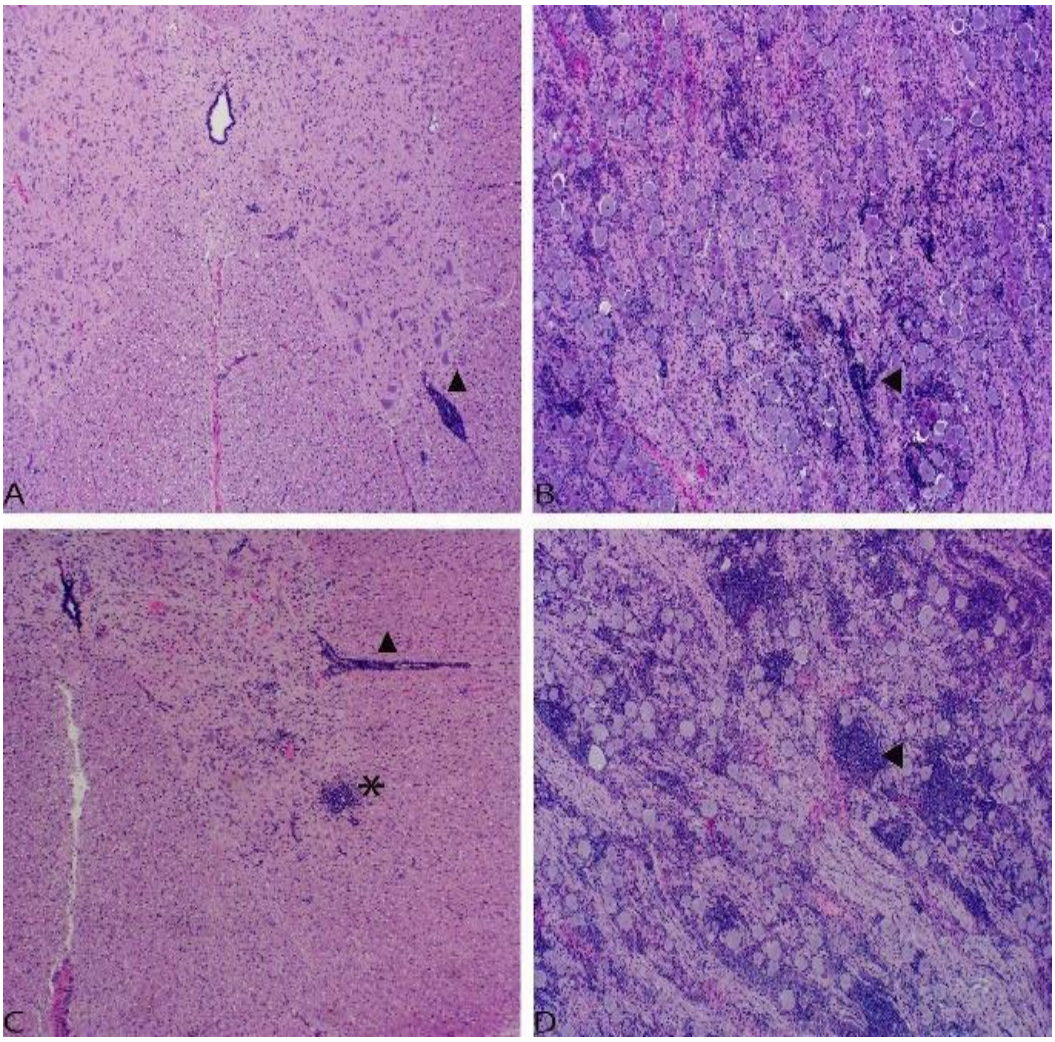

Figure S1: Pig #72, inoculated with PTV-2 (DPI 11). Lymphoplasmacytic myelitis with expansion of the Virchow-Robin spaces (▲) and multifocal areas of gliosis (*) in the cervical spinal cord (A) and lymphoplasmacytic ganglioneuritis(B). Pig #70 inoculated with PTV-11 (DPI 11) Lymphoplasmacytic myelitis with expansion of the Virchow-Robin spaces (▲) and multifocal areas of gliosis (*) in the thoracic spinal cord(C) and lymphoplasmacytic ganglioneuritis (◄) in spinal ganglion(D).

Supplemental Video 1. Hind limb ataxia, PTV-2-inoculate, pig #72, day post-inoculation 16. **Supplemental Video 2.** Paresis, PTV-11-inoculated, pig #70, day post-inoculation 10. (Videos available at www.proquest.com)

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Author Contributions: P.A., G.S., K.S. and B.A. conceived and designed the experiments; F.M.F., P.A, B.A., D.M., K.S., G.S., performed the experiments; F.M.F., P.A., B.A., K.Y., P.P., J.Q., Q.C. and K.S. analyzed the data; J.Z., K.Y. and P.P. contributed reagents/materials/analysis tools; F.M.F. and B.A. wrote the paper.

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CHAPTER 4. DETECTION OF *TESCHOVIRUS A* SEROTYPE 2 AND 11 BY RT-qPCR IN SAMPLES FROM EXPERIMENTALLY INOCULATED CESAREAN-DERIVED COLOSTRUM-DEPRIVED PIGS WITH TESCHOVIRUS ENCEPHALOMYELITIS.

Abstract. Teschovirus encephalomyelitis is clinically characterized by paresis, paralysis, and ataxia and is caused by *Teschovirus A* (formally *porcine teschovirus* (PTV)). Historically, PTV serotype 1 has been recognized as one of the most pathogenic serotypes, although other serotypes cause disease. In a previous study, 5-week-old cesarean-derived colostrum-deprived piglets developed encephalomyelitis following intravenous inoculation of strain PTV-2 USA/IA65463/2014 (n=7) or PTV-11 USA/IA09592/2013 (n=7). A real time qRT -PCR was used to detect PTV nucleic acids in various sample types from this study. Of 154 samples tested, PTV RNA was detected in 97 samples (63%). PTV was detected in 100% of samples from the cervical spinal cord, lumbar spinal cord, and mesenteric lymph node and 93% of samples from the thoracic spinal cord, spleen, and tracheobronchial lymph node. PTV was detected less commonly in the cerebrum (43%), lung and liver (21%), and heart and kidney (14%). The mean Cq value of tissue samples was 32.31 compared to a mean Cq of 22.86 and 32.46 in fecal and sera samples, respectively. Shedding of PTV was detected in feces of all inoculated piglets at 4 days post inoculation (DPI), and all live animals continued to shed until euthanasia at 21 DPI. Viremia was detected in all PTV inoculated animals at 4 DPI; however, viremia was inconsistently detected in different animals until the termination of the study. In this study, we quantified the detection of PTV nucleic acids in feces, serum, and various sample types and identified that cerebrum, while part of the central nervous system, may not be the best sample type to detect PTV in animals with TE. This is the first study assessing viral shedding,

viremia and distribution by qRT-PCR to detect PTV-2 and PTV-11 in experimentally infected pigs with Teschovirus encephalomyelitis.

Introduction

Teschovirus encephalomyelitis (TE), previously Teschen or Talfan disease, is a neurologic condition of pigs commonly characterized by locomotor disturbances including ataxia, paresis, and/or paralysis [3]. Teschen disease (also known as Klobouk's disease) was first recognized in the Czech Republic in 1929 as a fatal encephalomyelitis of pigs caused by a highly pathogenic strain of *Teschovirus A* (formerly named *Porcine enterovirus* and then *Porcine teschovirus*)[91]. *Teschovirus A* consists of 13 (sero)types, *porcine teschovirus* (PTV) 1 to 13 and is a single-stranded, linear, non-segmented RNA virus of the genus *Teschovirus*, family *Picornaviridae*[1]. The virus possesses a 28-30 nm icosahedral cáspside enclosing its genome. The positive-sense single-stranded linear RNA genome is approximately 7.8 kb long and encodes a single mRNA transcript [4,5]. Only one open reading frame is described, encoding a polypeptide that is cleaved post-translationally in the different structural and non-structural proteins [6].

Characteristic histologic lesions caused by PTV infections that result in TE include expansion of Virchow-Robin spaces by lymphocytes, plasma cells, and macrophages, multifocal areas of gliosis, and neuronal necrosis with satellitosis commonly in the spinal cord and brainstem and less commonly in the cerebrum [7,8,9,10]. Historically, PTV-1 has been recognized as one of the most pathogenic serotypes, although other serotypes have caused TE [11,12]. Hence, most information about the pathogenicity, epidemiology and control of disease relies on data from studies involving PTV-1. In a recent study, PTV-2 USA/IA65463/2014 and

PTV-11 USA/IA09592/2013 were shown to cause lesions consistent with TE in all inoculated cesarean-derived colostrum-deprived pigs and PTV was detected in the central nervous system tissue of affected animals. To the authors' knowledge, there is no information available in the scientific literature about the viral distribution, viral shedding in feces, or viremia of PTV serotypes 2 and 11 in experimentally infected animals. Therefore, the objective of this study was to describe the detection of PTV in multiple samples types, feces, and serum using a qRT-PCR in pigs with TE.

Materials and methods

Samples. In a previous experiment, eighteen colostrum deprived cesarean derived 5-week-old pigs were intravenously inoculated with one of two (sero)types of *Teschovirus A* or cell culture media. Pigs were divided into three groups and housed separately by inoculum: Minimum essential media (n=4), strain PTV-2 USA/IA65463/2014 (n=7) or strain PTV-11 USA/IA09592/2013 (n=7) (GenBank accession KY594021 and KY594022, respectively). Sera and fecal swabs were collected at day post inoculation (DPI) 0, 4, 7, 11 and 21 from all live animals. Animals were euthanized after showing clinical signs. Eight animals were euthanized at DPI 11 (control group=2, PTV-2 group=2, PTV-11 group=4), one animal from the PTV-2 group at DPI 16, and nine animals at DPI 21 (control group=2, PTV-2 group=4, PTV-11 group=3). At time of necropsy, the cerebrum; sections of cervical, thoracic, and lumbar spinal cord; heart, lung, liver, kidney, spleen, and mesenteric and tracheobronchial lymph nodes were collected. All samples were stored at -80^{0}C until further processing.

Reverse-transcription quantitative polymerase chain reaction. For the extraction of nucleic acids, 1 gram of the above-mentioned tissue samples from each animal was homogenized with 10 mL of Earl's minimum media. For fecal swabs, feces were collected at each time point an immersed in 1ml of PBS. Blood samples were collected at each time point and centrifuged at 2400 *rpm* for 10 minutes and serum was collected. Briefly, aliquots of 50 µl of sample were treated with MagMAX™-96 Total RNA Isolation Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and processed in a KingFisher™ Flex Purification System (Thermo Fisher Scientific) following the instructions of the manufacturer. Primer and probe combinations for 5' Taq nuclease assay using fluorescent 3' minor groove binding DNA probe were designed targeting the 5' UTR. Primer and probe sequences are: PTV Forward primer: GGTGGCGACAGRGTACAGA, PTV Reverse primer: CCTGCATTCCCRTACAGGAACT, PTV Probe: FAM- TGCRTTGCATATCCCTAG-MGB-BHQ. Amplification was carried out with a commercial RT-PCR amplification kit (QuantiTect Virus + ROX Vial Kit, Qiagen), according to the manufacturer's instructions. The final mastermix consisted of 5 µl of template and 20 µl of RT-PCR mix (5 µl of 5x QuantiTect Virus Master Mix, 0.25 µl of QuantiTect Virus RT Mix, 1.0 µl XENO LIZ internal control reagent, Life Technologies, 0.4 µM forward and reverse primers; fluorogenic TaqMan MGB probe, to a final concentration of 0.2 µm; and RNase-free water up to 20 µl). The reaction consisted of an RT step at 50°C for 20 min, followed by 5 min at 95°C (hot start) and 40 cycles of 15 s at 95°C and 1 min at 60°C. Samples in which PTV RNA was not detected after 40 cycles were considered negative.

Results

RT-qPCR Results.

PTV-2-inoculated group

Percent positive and mean Cq by sample type is presented in Figure 1. Results of the RT-qPCR by animal ID and sample type are presented in Table 1. Briefly, PTV was detected in all (7/7; 100%) cervical, thoracic, and lumbar spinal cord and mesenteric lymph node samples (mean Cq range: 28.30- 31.09), six (86%) tracheobronchial lymph node and spleen samples (mean Cq range: 32.50-32.88), two (28%) liver samples (mean Cq: 37.04), and one (14%) cerebrum, lung, and kidney sample (mean Cq range: 32.59-36.68). PTV was not detected in the heart. PTV was not detected in the serum at DPI 0 (Figure 2). PTV was detected in all serum samples at DPI 4 (mean Cq: 30.58). By DPI 7, only one animal (14%) had detectable levels of PTV RNA in serum. PTV was not in detected in the feces of any animal at DPI 0. At DPI 4 and all remaining time points, all live animals had detectable levels of PTV in feces (Figure 3; mean Cq range: 21.13-26.16).

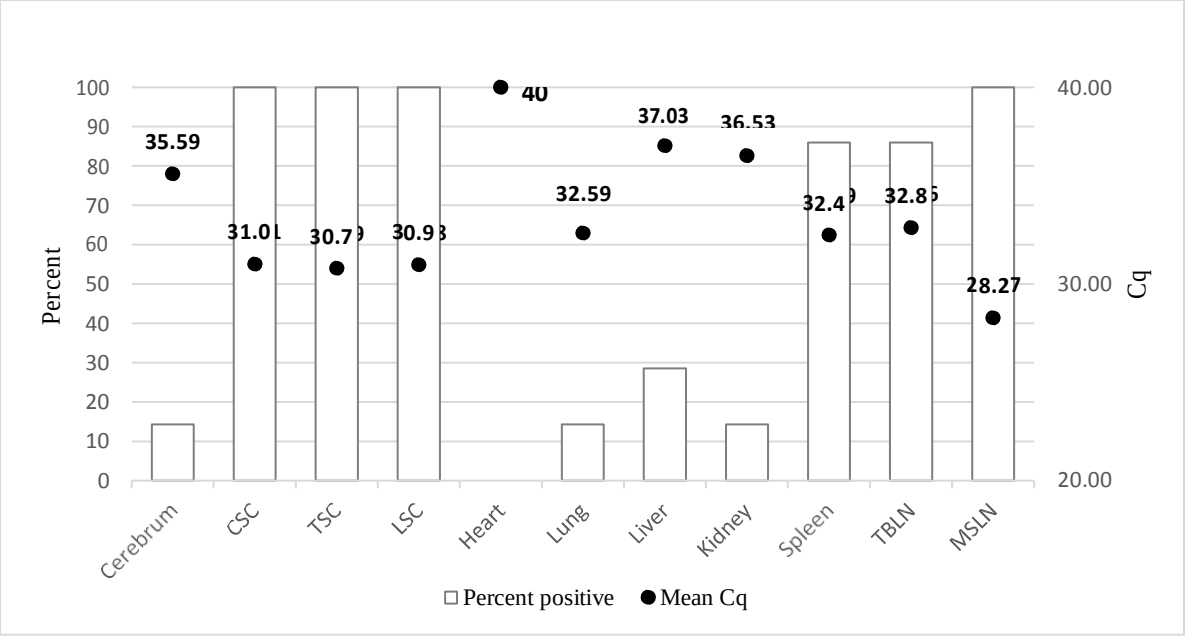

Figure 1. PTV-2 Percentage of positive samples and mean Cq value by tissue sample.

Table 1. RT-qPCR Results by Animal and Tissue Sample Type. Detection of *Teschovirus A* RNA by qRT-RCR targeting the 5'UTR region in various samples from control and inoculated piglets.

Group	*Animal ID*	Cerebrum	CSC[b]	TSC[c]	LSC[d]	Heart	Lung	Liver	Kidney	Spleen	TBLN[e]	MSLN[f]	
Control[a]	62	U	U	U	U	U	U	U	U	U	U	U	
	63	U	U	U	U	U	U	U	U	U	U	U	
	66	U	U	U	U	U	U	U	U	U	U	U	
	69	U	U	U	U	U	U	U	U	U	U	U	
PTV-2	61	U	34.45	35.98	32.67	U	U	U	U	31.17	31.24	29.54	
	67	U	28.64	29.23	28.47	U	U	U	U	33.3	31.91	29.54	
	68	U	34.38	33.29	35.65	U	U	37.59	U	32.95	33.94	27.95	
	72	35.59	30.24	30.65	30.28	U	U	U	U	32.83	34.03	29.16	
	73	U	33.22	34.18	34.81	U	U	U	U	32.79	U	28.62	
	74	U	25.74	22.59	24.32	U	U	36.48	36.68	31.93	32.78	26.97	
	76	U	31.47	31.68	32.25	U	32.59	U	U	U	33.37	26.3	
PTV-11	64	U	33.45	34.81	31.18	34.68	35.75	U	U	29.85	30.35	30.09	
	65	31.63	26.39	27.59	25.93	U	U	34.25	34.64	32.87	32.35	31.42	
	70	28.18	25.24	26.28	26.35	U	36.05	U	U	32.57	33.51	30.54	
	71	32.84	32.61	32.86	34.88	34.41	U	U	U	33.4	34.69	27.83	
	75	31.42	25.11	26.5	25.87	U	U	U	U	32.84	33.32	30.3	
	77	30.87	26.54	27.13	23.31	U	U	U	U	31.64	32.82	30.32	
	78	U	31.33	U	33.65	U	U	U	U	33.29	33.53	28.35	

[a] Inoculated with 3ml of minimum essential media (MEM).
[b] CSC = Cervical spinal cord. [c]
TSC = Thoracic spinal cord. [d]
LSC = Lumbar spinal cord.
[e] TBLN = Tracheobronchial lymph node. [f]
MSLN= Mesenteric lymph node.
U indicates "undetected" following 40 cycles.
ND = Not done.

PTV-11-inoculated group

Percent positive and mean Cq by sample type is presented in Figure 4. Results of the RT-qPCR by animal ID and sample type are presented in Table 1. Briefly, PTV was detected in all (7/7; 100%) cervical and lumbar spinal cord, spleen, and tracheobronchial and mesenteric lymph node samples (mean Cq range: 28.67-32.94). PTV was detected in six (86%) thoracic spinal cord (mean Cq: 29.20), five (71 %) cerebrum (mean Cq; 30.99), two (28%) lung and heart sample (mean Cq: 35.90 and 34.55, respectively) and one (14%) kidney and liver sample (mean Cq: 34.64 and 34.25). Serum and fecal RT-qPCR results by animal and day post inoculation are presented in Table 2. Briefly, PTV was not detected in the serum at DPI 0 (Figure 2). PTV was detected in all serum samples at DPI 4 (mean Cq: 31.85). At DPI 7, 11, and 21, two of 7 samples were positive (mean Cq range: 34.88-35.18). Positive samples were from different animals at each time point. At DPI 0, PTV was not detected in feces. PTV was detected in all available samples until DPI 21 (Figure 3; mean Cq range: 20.96-26.02).

Sham-inoculated group

Nucleic acids of PTV were not detected in any sample from control animals.

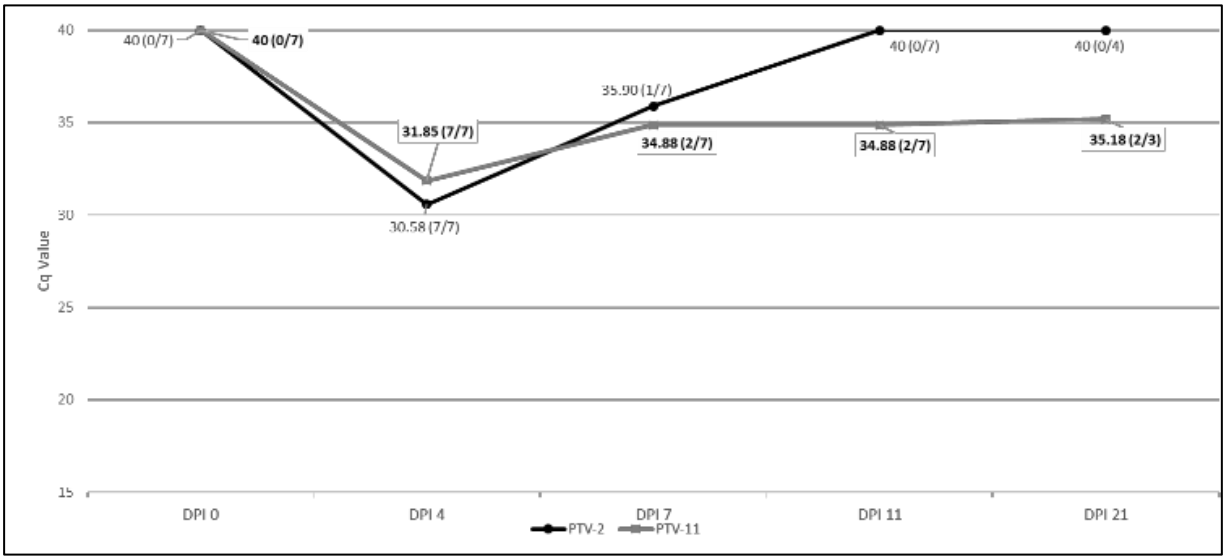

Figure 2. Mean serum Cq by serotype.

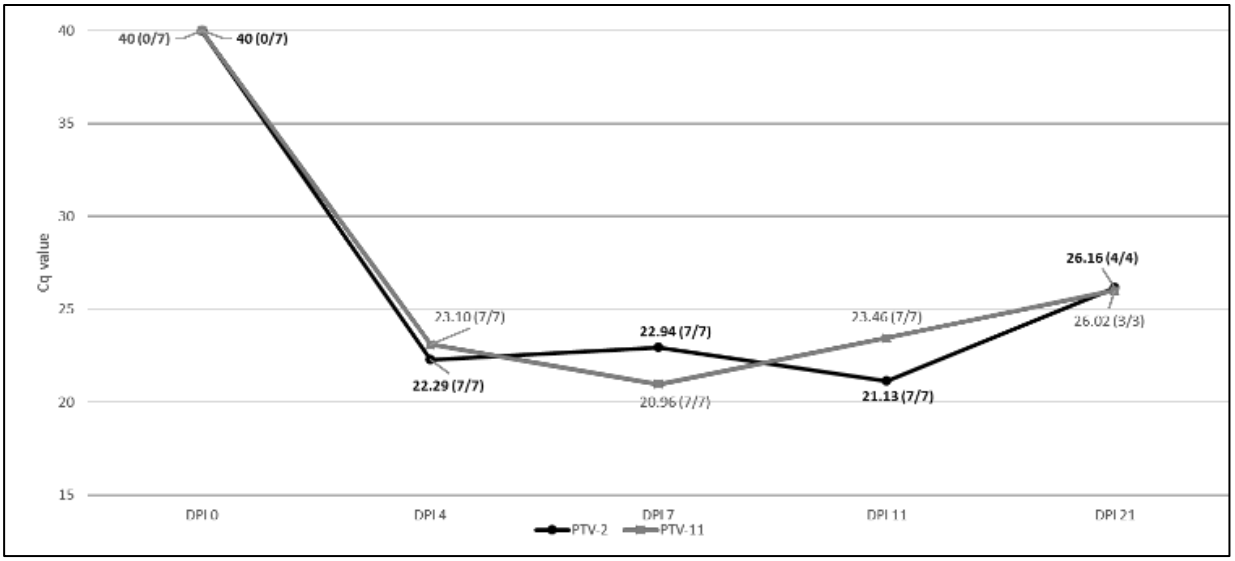

Figure 2. Mean fecal Cq by serotype

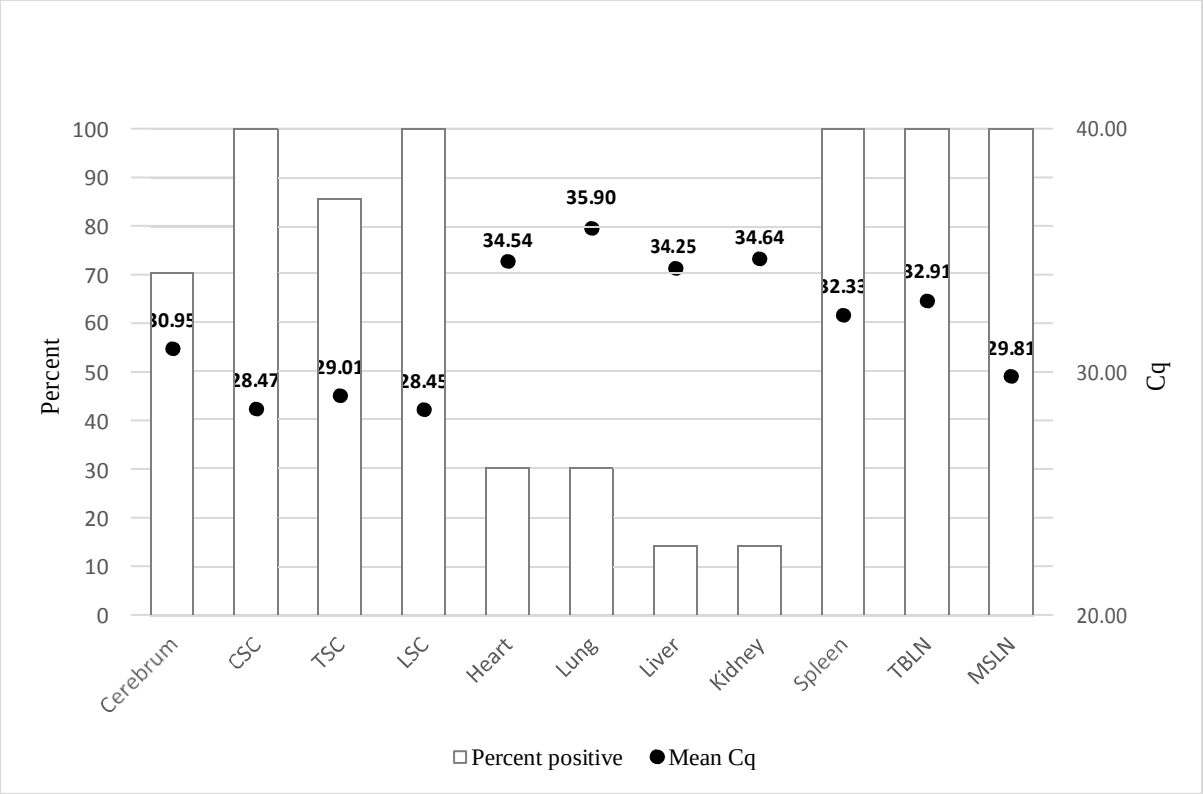

Figure 4. PTV-11 Percentage of positive samples and mean Cq value by tissue sample.

Table 2. qRT-PCR Serum and Fecal Results by Animal and Day Post Inoculation. Detection of *Teschovirus A* RNA by qRT-RCR targeting the 5'UTR region from control and inoculated piglets.

Group	*Animal ID*	Feces					Serum				
		DPI 0	**DPI 4**	**DPI 7**	**DPI 11**	**DPI 21**	**DPI 0**	**DPI 4**	**DPI 7**	**DPI 11**	**DPI 21**
Control	62	U	U	U	U	U	U	U	U	U	U
	63	U	U	U	U	U	U	U	U	U	U
	66	U	U	U	U	U	U	U	U	U	U
	69	U	U	U	U	U	U	U	U	U	U
PTV-2	61	U	26.65	20.73	24.04	29.91	U	27.11	U	U	U
	67	U	21.66	20.53	18.63	ND	U	30.89	U	U	ND
	68	U	20.4	21.49	23.11	29.74	U	30.67	U	U	U
	72	U	24.17	23.51	21.01	20.32	U	33.14	U	U	ND
	73	U	21.59	20.49	21.52	26.46	U	29.8	U	U	U
	74	U	22.64	31.15	19.54	ND	U	30.42	35.9	U	U
	76	U	19.62	24.35	20.59	25.62	U	32.43	U	U	U
PTV-11	64	U	24.4	20.65	24.01	27.98	U	28.33	34.71	U	U
	65	U	22.47	20.06	22.03	ND	U	30.98	U	U	ND
	70	U	24.4	19.79	24.47	ND	U	34.26	U	U	ND
	71	U	21.89	21.24	20.84	26.94	U	34.71	U	U	34.77
	75	U	20.15	21.27	20.85	ND	U	30.3	U	35.36	ND
	77	U	24.21	21.21	32.19	ND	U	31.91	U	34.4	ND
	78	U	24.58	22.59	21.59	23.36	U	32.98	35.05	U	35.6

[a] Inoculated with 3ml of minimum essential media (MEM).
U indicates "undetected" following 40 cycles.
ND = Not done.

Discussion

Viral shedding in feces was detected at 4 DPI in all animals prior to clinical signs of TE, which were first noted in the PTV-11-inculated group at 9 DPI and 12 DPI in the PTV-2-inouclated group. All live animals with and without clinical signs consistent with TE shed PTV through the termination of the study at DPI 21.

Fecal Cq values were approximately 10-100 logs lower in comparison to the mesenteric lymph node which is the next lowest mean Cq value. These findings are similar to other studies in which PTV-1 was detected by RT-PCR in the large intestine of animals with TE at DPI 6, 13, 20, and 28 and virus isolation at DPI 21 [13] and detection of PTV-2 by virus isolation at DPI 21 in animals with and without TE. Dardiri *et al.* detected PTV-1 by virus isolation at DPI 2 in a pig that developed TE following intracranial and intranasal inoculation [14].

Viremia was detected at 4 DPI in all virus inoculated animals; however by DPI 7 only three animals had detectable levels of PTV in serum. At DPI 11 and 21 two animals at each time point had detectable levels of PTV in serum. Based on these findings, it appears that viremia is inconsistently detected in animals with histologic evidence of TE. Others have detected PTV-1 in serum at necropsy consistently in animals with TE at DPI 6 and 13 and less consistently by DPI 20 with PTV-1 not being detected in serum by DPI 28 [15].

Teschovirus A nucleic acid was commonly detected in the spinal cord of viral inoculated animals and less common in the cerebrum of animals inoculated with PTV-2. In both viral inoculated groups the mesenteric lymph node had the lowest Cq value of any tissue sample. One possible explanation could be migration of lymphoid cells carrying PTV from the intestine to the

mesenteric lymph node. Apart from samples in the spinal cord, PTV was most consistently detected in lymphoid tissues including spleen, tracheobronchial lymph node and mesenteric lymph node. In the evaluation of endemically infected animals in which the status of clinical signs consistent with TE are not available, Chui *et al.* also found that lymph node samples yielded the highest relative viral load and cerebrum (pooled cranial and medial sections) yielded the lowest relative viral load [16]. Dardiri *et al.* also found a significantly lower level of virus in the thalamus, motor cortex, and olfactory bulb compared to the cervical and thoracic spinal cord.

The distribution and higher presence of PTV RNA in spinal cord samples could reflect the findings made by several authors that specific histological lesions are more frequently found in spinal cord than in cerebrum. Similar to other studies in animals with TE, PTV was detected less commonly in the liver and kidney [14,15]. Unlike studies using PTV-1 strains in which 91% to 100% of animals with TE were positive by PCR or virus isolation, PTV nucleic acid was detected less frequently (14% to 30%) in the lung in animals with TE following PTV-2 or PTV-11 inoculation [14,15]. This may be a result of assay sensitivity or serotype variation.

In conclusion, viral shedding of PTV in feces can be detected by RT-qPCR consistently in all animals prior to the development of clinical signs of TE; however, viremia is less consistently detected by RT-qPCR even in animals with clinical signs of TE. Although viral nucleic acid was detected in a vast majority of spinal cord and lymphoid samples, cerebrum while part of the central nervous system, may not be the best sample type to detect PTV in animals with TE.

CHAPTER 6. SUMMARY AND CONCLUSION

The purpose of this thesis was to determine and corroborate the neuropathogenicity of two wild strains of *Teschovirus A* recently identified. These two strains, PTV-2 USA/IA65463/2014 and PTV-11 USA/IA09592/2013 were isolated from clinical cases submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL).

A literature review chapter initiates this thesis followed by a short chapter where cases of Teschovirus encephalomyelitis diagnosed at ISUVDL during the years 2013-2016 were identified. The next chapter consist in the experimental inoculation of both viruses in susceptible animals as the results and conclusions from this experiment, and a final research chapter where the distribution and detection of PTV nucleic acids is assessed and quantified.

The main objective of this thesis, to induce Teschovirus encephalomyelitis in experimental animals with wild-type strains PTV-2 USA/IA65463/2014 and PTV-11 USA/IA09592/2013 was successfully achieved.

A porcine model was developed to assess the neuropathogenicity of both (sero) types. The porcine model adopted for this research demonstrated to be a successful platform to induce experimental disease. Animals in both inoculated groups developed disease and the virus was later recovered from affected tissues. It is relevant to remark that this if the first description of PTV serotype 11 in the United States and further research should be conducted to elucidate how this pathogen could affect swine herds.

The detection of PTV nucleic acids in different sample types allowed quantification of this pathogen in CNS tissue corroborating its neuropathogenicity and permitted to identify sample types suitable for diagnosis.

The findings from this study are limited due to the small sample size used in the experiments. This is the major limitation of this thesis and the conclusions as indications from this work should be interpreted contemplating this factor.

Thus far, the epidemiology of this pathogen in the U.S. as the differences between serotypes is not completely understood and possible areas for further investigation should contemplate the diagnosis of other serotypes as the presence of novel serotypes in the swine industry.

REFERENCES

1. International Committee on Taxonomy of Viruses.; King, A.M.Q. *Virus taxonomy : Classification and nomenclature of viruses : Ninth report of the international committee on taxonomy of viruses*. Academic Press: London ; Waltham, MA, 2012; p x, 1327 p.
2. Jeffrey J. Zimmerman (Editor), L.A.K.E., Alejandro Ramirez (Editor), Kent J. Schwartz (Editor), Gregory W. Stevenson (Editor). *Diseases of swine, 10th edition*. 2012; p 1008.
3. OIE. *Manual of diagnostic tests and vaccines for terrestrial animals 2016*. 8th Edition ed.; OIE: 2016; Vol. 2.
4. Dunne, H.W.; Leman, A.D. *Diseases of swine*. 4th ed.; Iowa State Univ. Press: Ames, Iowa, 1975; p xvi, 1212 p.
5. Buitrago, D.; Cano-Gomez, C.; Aguero, M.; Fernandez-Pacheco, P.; Gomez-Tejedor, C.; Jimenez-Clavero, M.A. A survey of porcine picornaviruses and adenoviruses in fecal samples in spain. *J Vet Diagn Invest* **2010**, *22*, 763-766.
6. Boros, Á.; Pankovics, P.; Reuter, G. Characterization of a novel porcine enterovirus in domestic pig in hungary. *Infection, Genetics and Evolution* **2011**, *11*, 1096-1102.
7. Trefny, L. Massive illness of swine in teschen area. *Zveroleki Obzori* **1930**, *23*, 235-236.
8. Pavlas, M. Antonin klobouk, biography. *Hist Med Vet* **2006**, *31*, 92-93.
9. A., K. Aetiology of the so-called teschen disease – encephalomyelitis enzootica suum. *Zvěrolékařské rozpravy* **1933**, *VII*, 85-265.
10. Harding, J.D.J.; Done, J.T.; Kershaw, G.F. A transmissible polioencephalomyelitis of pigs (talfan disease). *Vet Rec* **1957**, *69*.
11. Thordal-Christensen, A. *A study of benign enzootic paresis of pigs in denmark*. Carl Fr. Mortensen: 1959.
12. Mayr, A.; Federal Research Institute for Virus Diseases of Animals, T., Germany. The virus of teschen disease. *Annals of the New York Academy of Sciences* **2016**, *101*, 423-427.
13. *Fao/oie animal health yearbook*. 1959.
14. Chaproniere, D.M.; Done, J.T.; Andrewes, C.H. Comparative serological studies on talfan and teschen diseases and similar conditions. *Br J Exp Pathol* **1958**, *39*, 74-77.
15. L'Ecuyer, C.; Greig, A.S. Serological and biological studies on porcine enteroviruses isolated in canada. *Can Vet J* **1966**, *7*, 148-154.
16. Dunne, H.W.; Kradel, D.C.; Clark, C.D.; Bubash, G.R.; Ammerman, E. Porcine enteroviruses: A serologic comparison of thirty-eight pennsylvania isolates with other reported north american strains, teschen, talfan, and t80 serums--a progress report. *Am J Vet Res* **1967**, *28*, 557-568.
17. Dunne, H.W.; Wang, J.T.; Ammerman, E.H. Classification of north american porcine enteroviruses: A comparison with european and japanese strains 1. *Infect Immun* **1971**, *4*, 619-631.
18. Thorsen, J.; Macpherson, L.W. A study of porcine enteroviruses isolated from swine in the toronto area: 1. Isolation and serological grouping of viruses. *Can J Comp Med Vet Sci* **1966**, *30*, 308-310.

19. Wang, J.T.; Dunne, H.W. Comparison of antiserums produced in chickens, rabbits, and specific-pathogen-free pigs for the typing of porcine enteroviruses. *Am J Vet Res* **1972**, *33*, 811-816.
20. Christov, S. Serological classification of swine enteroviruses. *Zentralbl Veterinarmed B* **1966**, *13*, 31-36.
21. Betts, A.O.; Lamont, P.H.; Kelly, D.F. Porcine enteroviruses other than the virus of teschen disease. *Ann N Y Acad Sci* **1962**, *101*, 428-435.
22. Zoletto, R. Caratteristiche differenzialii degli enterovirus suini. *Vet. Ital* **1965**, *6*, 3-20.
23. virus, C.i.d.n.d.; Wildy, P. *Classification and nomenclature of viruses: First report of the international committee on nomenclautre of viruses*. S. Karger: 1971.
24. Fenner, F. Classification and nomenclature of viruses. Second report of the international committee on taxonomy of viruses. *Intervirology* **1976**, *7*, 1-115.
25. Knowles, N.J.; Buckley, L.S.; Pereira, H.G. Classification of porcine enteroviruses by antigenic analysis and cytopathic effects in tissue culture: Description of 3 new serotypes. *Arch Virol* **1979**, *62*.
26. Auerbach, J.; Prager, D.; Neuhaus, S.; Loss, U.; Witte, K.H. Grouping of porcine enteroviruses by indirect immunofluorescence and description of two new serotypes. *Zentralblatt für Veterinärmedizin. Reihe B* **1994**, *41*.
27. Chaproniere, D.M.; Done, J.T.; Andrewes, C.H. Comparative serological studies on talfan and teschen diseases and similiar conditions. *Br J Exp Pathol* **1958**, *39*, 74-77.
28. Odend'Hal, S. Teschen disease virus. In *The geographical distribution of animal viral diseases*, Academic Press: 1983; pp 413-415.
29. Pringle, C.R. Virus taxonomy at the xith international congress of virology, sydney, australia, 1999. *Archives of Virology* **1999**, *144*, 2065-2070.
30. Fauquet, C.M.; Mayo, M.A. The 7th ictv report. *Arch Virol* **2001**, *146*, 189-194.
31. Krumbholz, A.; Dauber, M.; Henke, A.; Birch-Hirschfeld, E.; Knowles, N.J.; Stelzner, A.; Zell, R. Sequencing of porcine enterovirus groups ii and iii reveals unique features of both virus groups. In *J virol*, 2002; Vol. 76, pp 5813-5821.
32. Zell, R.; Seitz, S.; Henke, A.; Munder, T.; Wutzler, P. Linkage map of protein-protein interactions of porcine teschovirus. *J Gen Virol* **2005**, *86*, 2763-2768.
33. Pathak, H.B.; Arnold, J.J.; Wiegand, P.N.; Hargittai, M.R.; Cameron, C.E. Picornavirus genome replication: Assembly and organization of the vpg uridylylation ribonucleoprotein (initiation) complex. *J Biol Chem* **2007**, *282*, 16202-16213.
34. Kaku, Y.; Chard, L.S.; Inoue, T.; Belsham, G.J. Unique characteristics of a picornavirus internal ribosome entry site from the porcine teschovirus-1 talfan. *J Virol* **2002**, *76*, 11721-11728.
35. Luke, G.A.; de Felipe, P.; Lukashev, A.; Kallioinen, S.E.; Bruno, E.A.; Ryan, M.D. Occurrence, function and evolutionary origins of '2a-like' sequences in virus genomes. In *J gen virol*, 2008; Vol. 89, pp 1036-1042.
36. Doherty, M.; Todd, D.; McFerran, N.; Hoey, E.M. Sequence analysis of a porcine enterovirus serotype 1 isolate: Relationships with other picornaviruses. **1999**.
37. Agirre, A.; Barco, A.; Carrasco, L.; Nieva, J.L. Viroporin-mediated membrane permeabilization. **2002**.

38. Vignuzzi, M.; Stone, J.K.; Arnold, J.J.; Cameron, C.E.; Andino, R. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* **2006**, *439*, 344-348.
39. Wang, B.; Tian, Z.J.; Gong, D.Q.; Li, D.Y.; Wang, Y.; Chen, J.Z.; An, T.Q.; Peng, J.M.; Tong, G.Z. Isolation of serotype 2 porcine teschovirus in china: Evidence of natural recombination. *Vet Microbiol* **2010**, *146*, 138-143.
40. Derbyshire, J.B.; Collins, A.P. An experimental epidemiological study of talfan virus infection in pigs. *Journal of Comparative Pathology* **1972**, *82*, 315-322.
41. Hajek, P.; Mandel, L. The antibody response in newborn precolostral germfree piglets following the peroral monocontamination with attenuated teschen disease virus (tdv). *Folia Microbiol (Praha)* **1971**, *16*, 58-61.
42. Edington, N.; Christofinis, G.J.; Betts, A.O. Pathogenicity of talfan and konratice strains of teschen virus in gnotobiotic pigs. *Journal of Comparative Pathology* **1972**, *82*, 393-399.
43. Brundage, L.J.; Derbyshire, J.B.; Wilkie, B.N. Cell mediated responses in a porcine enterovirus infection in piglets. *Can J Comp Med* **1980**, *44*, 61-69.
44. Dardiri, A.H.; Seibold, H.R.; DeLay, P.D. The response of colostrum-deprived, specific pathögen-free pigs to experimental infection with teschen disease virus. *Can J Comp Med Vet Sci* **1966**, *30*, 71-81.
45. An in vivo characterization of colostrum protein uptake in porcine gut during early lactation. **2011**, *74*, 101–109.
46. Hajek, P.; Mandel, L.; Smolova, M. Neutralizing antibody formation in newborn colostrum-deprived germfree piglets infected per os with teschen disease virus. *Acta Virol* **1971**, *15*, 430.
47. Singh, K.V.; Bohl, E.H. The pattern of enteroviral infections in a herd of swine. *Can J Comp Med* **1972**, *36*, 243-248.
48. Danielsen, M.; Department of Animal Health, W.a.N., Danish Institute of Agricultural Sciences, Tjele, Denmark; Department of Biochemistry and Molecular Biology, U.o.S.D., Odense M, Denmark; Thymann, T.; Department of Human Nutrition, R.V.a.A.U., Frederiksberg C, Denmark; Jensen, B.B.; Department of Animal Health, W.a.N., Danish Institute of Agricultural Sciences, Tjele, Denmark; Jensen, O.N.; Department of Biochemistry and Molecular Biology, U.o.S.D., Odense M, Denmark; Sangild, P.T., *et al.* Proteome profiles of mucosal immunoglobulin uptake in inflamed porcine gut. *PROTEOMICS* **2016**, *6*, 6588-6596.
49. Derbyshire, J.B. Enterovirus neutralizing activity in the gastrointestinal tract of piglets. *Can J Comp Med* **1974**, *38*, 425-429.
50. Humoral and cellular factors of maternal immunity in swine. **2009**, *33*, 384–393.
51. Hazlett, D.T.G.; Derbyshire, J.B. Broad specificity of gastrointestinal antibodies following vaccination of piglets with a porcine enterovirus. *Journal of Comparative Pathology* **1978**, *88*, 467-471.
52. Pogranichniy, R.M.; Janke, B.H.; Gillespie, T.G.; Yoon, K.J. A prolonged outbreak of polioencephalomyelitis due to infection with a group i porcine enterovirus. *J Vet Diagn Invest* **2003**, *15*, 191-194.
53. Hazlett, D.T.; Derbyshire, J.B. The protective effect of two porcine enterovirus vaccines in swine. *Can J Comp Med* **1977**, *41*, 264-273.

54. Broad specificity of gastrointestinal antibodies following vaccination of piglets with a porcine enterovirus. **2016**.
55. Derbyshire, J.B. The interferon sensitivity of selected porcine viruses. *Can J Vet Res* **1989**, *53*, 52-55.
56. Chang, S.M.; Lee, C.S.K.N.U., Taegu (Korea R.). Dept. of Veterinary Medicine). Effect of dexamethasone on porcine enterovirus infections in pigs. *Korean Journal of Veterinary Research (Korea R.)* **1986**.
57. Derbyshire, J.B. The effect of immunosuppression with cyclophosphamide on an experimental porcine enterovirus infection in piglets. *Can J Comp Med* **1983**, *47*, 235-237.
58. Wang, L.; Ge, C.; Wang, D.; Li, Y.; Hu, J. The survey of porcine teschoviruses, porcine circovirus and porcine transmissible gastroenteritis virus infecting piglets in clinical specimens in china. *Trop Anim Health Prod* **2013**, *45*, 1087-1091.
59. Handke, M.; Engels, M.; Prohaska, S.; Keller, C.; Brugnera, E.; Sydler, T.; Sidler, X. [infection related fertility disorders in swiss pig breeding farms at the end of the postweaning multisystemic wasting syndrome (pmws) epizooty]. *Schweiz Arch Tierheilkd* **2012**, *154*, 437-444.
60. Chiu, S.C.; Hu, S.C.; Chang, C.C.; Chang, C.Y.; Huang, C.C.; Pang, V.F.; Wang, F.I. The role of porcine teschovirus in causing diseases in endemically infected pigs. *Vet Microbiol* **2012**, *161*, 88-95.
61. Takahashi, M.; Seimiya, Y.M.; Seki, Y.; Yamada, M. A piglet with concurrent polioencephalomyelitis due to porcine teschovirus and postweaning multisystemic wasting syndrome. *J Vet Med Sci* **2008**, *70*, 497-500.
62. Donin, D.G.; de Arruda Leme, R.; Alfieri, A.F.; Alberton, G.C.; Alfieri, A.A. First report of porcine teschovirus (ptv), porcine sapelovirus (psv) and enterovirus g (ev-g) in pig herds of brazil. *Trop Anim Health Prod* **2014**, *46*, 523-528.
63. Yang, S.; Wang, Y.; Shen, Q.; Zhang, W.; Hua, X. Prevalence of porcine enterovirus 9 in pigs in middle and eastern china. *Virology Journal* **2013**, *10*, 99.
64. Nix, W.A.; Khetsuriani, N.; Penaranda, S.; Maher, K.; Venczel, L.; Cselko, Z.; Freire, M.C.; Cisterna, D.; Lema, C.L.; Rosales, P., *et al.* Diversity of picornaviruses in rural bolivia. *J Gen Virol* **2013**, *94*, 2017-2028.
65. Janke, B.H.; Morehouse, L.G.; Solorzano, R.F. Single and mixed infections of neonatal pigs with rotaviruses and enteroviruses: Virological studies. *Can J Vet Res* **1988**, *52*, 360-363.
66. Janke, B.H.; Morehouse, L.G.; Solorzano, R.F. Single and mixed infections of neonatal pigs with rotaviruses and enteroviruses: Clinical signs and microscopic lesions. *Can J Vet Res* **1988**, *52*, 364-369.
67. Huck, R.A. Experimental disease produced in pigs following infection with the konratice and talfan strains of the teschen group of viruses. *J Comp Pathol* **1962**, *72*, 380-388.
68. Yamada, M.; Kozakura, R.; Kaku, Y.; Nakamura, K.; Yamamoto, Y.; Yoshii, M.; Miyazaki, A.; Tsunemitsu, H.; Narita, M. Immunohistochemical distribution of viral antigens in pigs naturally infected with porcine teschovirus. *J Vet Med Sci* **2008**, *70*, 305-308.
69. Chiu, S.C.; Yang, C.L.; Chen, Y.M.; Hu, S.C.; Chiu, K.C.; Lin, Y.C.; Chang, C.Y.; Wang, F.I. Multiple models of porcine teschovirus pathogenesis in endemically infected pigs. *Vet Microbiol* **2014**, *168*, 69-77.

70. Yamada, M.; Kozakura, R.; Nakamura, K.; Yamamoto, Y.; Yoshii, M.; Kaku, Y.; Miyazaki, A.; Tsunemitsu, H.; Narita, M. Pathological changes in pigs experimentally infected with porcine teschovirus. *Journal of Comparative Pathology* **2009**, *141*, 223-228.
71. Long, J.F.; Koestner, A.; Liss, L. Experimental porcine polioencephalomyelitis in germfree pigs. A silver carbonate study of neuronal degeneration and glial response. *Pathol Vet* **1967**, *4*, 186-198.
72. Manuelidis, E.E.; Sprinz, H.; Horstmann, D.M. Pathology of teschen disease (virus encephalomyelitis of swine). *Am J Pathol* **1954**, *30*, 567-597.
73. Serres, H. *Catalog record: La maladie de teschen. La maladie de talfan; picornaviroses porcines*. Paris, L'Expansion scientifique française, 1970; p 215 p.
74. Maes, D.; Van Soom, A.; Appeltant, R.; Arsenakis, I.; Nauwynck, H. Porcine semen as a vector for transmission of viral pathogens. *Theriogenology* **2016**, *85*, 27-38.
75. Tsai, A.T.; Kuo, C.C.; Kuo, Y.C.; Yang, J.L.; Chang, C.Y.; Wang, F.I. The urinary shedding of porcine teschovirus in endemic field situations. *Vet Microbiol* **2016**, *182*, 150-155.
76. Guerin, B.; Pozzi, N. Viruses in boar semen: Detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination. *Theriogenology* **2005**, *63*, 556-572.
77. Lin, W.; Cui, S.; Zell, R. Phylogeny and evolution of porcine teschovirus 8 isolated from pigs in china with reproductive failure. *Arch Virol* **2012**, *157*, 1387-1391.
78. Daniel Givens, M.; Marley, M.S.D. Infectious causes of embryonic and fetal mortality. *Theriogenology* **2008**, *70*, 270-285.
79. Chiu, S.-C. A revisit of porcine teschovirus pathogenesis in pigs. **2014**.
80. Long, J.F.; Koestner, A.; Kasza, L. Infectivity of three porcine polioencephalomyelitis viruses for germfree and pathogen-free pigs. *Am J Vet Res* **1966**, *27*, 274-279.
81. Bangari, D.S.; Pogranichniy, R.M.; Gillespie, T.; Stevenson, G.W. Genotyping of porcine teschovirus from nervous tissue of pigs with and without polioencephalomyelitis in indiana. *J Vet Diagn Invest* **2010**, *22*, 594-597.
82. Deng, M.Y.; Millien, M.; Jacques-Simon, R.; Flanagan, J.K.; Bracht, A.J.; Carrillo, C.; Barrette, R.W.; Fabian, A.; Mohamed, F.; Moran, K., *et al.* Diagnosis of porcine teschovirus encephalomyelitis in the republic of haiti. *J Vet Diagn Invest* **2012**, *24*, 671-678.
83. Chiu, S.-C.; Hu, S.-C.; Chang, C.-C.; Chang, C.-Y.; Huang, C.-C.; Pang, V.F.; Wang, F.-I. The role of porcine teschovirus in causing diseases in endemically infected pigs. *Veterinary Microbiology* **2012**, *161*, 88-95.
84. Hartmann, W. Foreign animal disease diagnostician investigation of a disease incident involving a central nervous system (cns) syndrome in finisher pigs. **2005**.
85. Salles, M.W.; Scholes, S.F.; Dauber, M.; Strebelow, G.; Wojnarowicz, C.; Hassard, L.; Acton, A.C.; Bollinger, T.K. Porcine teschovirus polioencephalomyelitis in western canada. *J Vet Diagn Invest* **2011**, *23*, 367-373.
86. Sibalin, M. Confirmation of the presence of the talfan disease in swedish pigs. *Arch Gesamte Virusforsch* **1962**, *12*, 76-88.
87. Yamada, M.; Kozakura, R.; Ikegami, R.; Nakamura, K.; Kaku, Y.; Yoshii, M.; Haritani, M. Enterovirus encephalomyelitis in pigs in japan caused by porcine teschovirus. *Vet Rec* **2004**, *155*, 304-306.

88. Kasza, L. Swine polioencephalomyelitis virus isolated from the brains and intestines of pigs. *Am J Vet Res* **1965**, *26*.
89. Yamada, M.; Miyazaki, A.; Yamamoto, Y.; Nakamura, K.; Ito, M.; Tsunemitsu, H.; Narita, M. Experimental teschovirus encephalomyelitis in gnotobiotic pigs. *Journal of Comparative Pathology* **2014**, *150*, 276-286.
90. Lomniczi, B. Is species specific resistance alterable by ethionine? Ii. Infection experiments on rats with teschen disease virus. *Z Immunitatsforsch Allerg Klin Immunol* **1967**, *132*, 486-494.
91. Kouba, V. Teschen disease (teschovirus encephalomyelitis) eradication in czechoslovakia: A historical report. *Veterinarni Medicina* **2009**, *54*, 550-560.
92. Handistatus ii multiannual animal disease status. http://web.oie.int/hs2/sit_mald_freq_pl.asp?c_cont=1&c_mald=81
93. Witte, K.H.; Auerbach, J.; Loss, K.U.; Neuhaus, S.; Prager, D. [typing of 17 porcine enterovirus isolates from polio encephalomyelitis cases during the years 1983-1991]. *Dtsch Tierarztl Wochenschr* **1994**, *101*, 482-484.
94. Mayr, A.; Bibrack, B.; Kublbeck, E. [serological studies on the occurrence of teschen-talfan-infections in pigs in bavaria]. *Zentralbl Veterinarmed B* **1971**, *18*, 505-516.
95. Nolewajka, E.; Szaflarski, J. [history of studies on the teschen disease virus (meningoencephalomyelitis enzootica suum) in poland]. *Pol Arch Weter* **1974**, *17*, 423-433.
96. Basso, W.; Handke, M.; Sydler, T.; Borel, N.; Grimm, F.; Sidler, X.; Deplazes, P. Involvement of toxoplasma gondii in reproductive disorders in swiss pig farms. *Parasitology International* **2015**, *64*, 157-160.
97. Sytiuk, M.P.; Spyrydonov, V.G.; Postoienko, V.O.; Melnychuk, S.D.; Nedosekov, V.V. Molecular and genetic characteristics isolates virus of porcine enzootic encephalomyelitis isolated from wild boar and domestic swine on the territory of ukraine. *Біологія тварин* **2016**, 133-142.
98. Metianu, T.; Atanasiu, P.; Lucas, A. Experimental study of teschen's disease. Isolation and identification of 2 strains of virus in france. *Bull Acad Vet Fr* **1970**, *43*, 151-159.
99. Sozzi, E.; Barbieri, I.; Lavazza, A.; Lelli, D.; Moreno, A.; Canelli, E.; Bugnetti, M.; Cordioli, P. Molecular characterization and phylogenetic analysis of vp1 of porcine enteric picornaviruses isolates in italy. *Transbound Emerg Dis* **2010**, *57*, 434-442.
100. Pini, A.; Smit, G. Incidence and distribution of neutralizing antibodies to porcine enteroviruses in southern africa. *J S Afr Vet Assoc* **1973**, *44*, 247-250.
101. Feng, L.; Shi, H.Y.; Liu, S.W.; Wu, B.P.; Chen, J.F.; Sun, D.B.; Tong, Y.E.; Fu, M.S.; Wang, Y.F.; Tong, G.Z. Isolation and molecular characterization of a porcine teschovirus 1 isolate from china. *Acta Virol* **2007**, *51*, 7-11.
102. The prevalence of porcine teschovirus in the pig population in northeast of china. **2013**, *193*, 209–214.
103. Hsu, B.M.; Chen, C.H.; Wan, M.T. Prevalence of enteroviruses in hot spring recreation areas of taiwan. *FEMS Immunol Med Microbiol* **2008**, *52*.
104. Ventura, A.; Gonzalez, W.; Barrette, R.; Swenson, S.; Bracht, A.; Rowland, J.; Fabian, A.; Moran, K.; Mohamed, F.; O'Hearn, E., *et al.* Virus and antibody diagnostics for swine samples of the dominican republic collected in regions near the border to haiti. *ISRN Virology* **2013**, *2013*, 1-7.

105. Kresse, J.I.; Snyder, M.L.; Fynskov, P.H.; Stewart, W.C. Isolation of a new porcine enterovirus in the united states. *Can J Comp Med* **1977**, *41*, 355-356.
106. Burgess, G.W. The isolation and characterization of porcine enteroviruses. http://dx.doi.org/10.1080/00480169.1977.34397 **2011**.
107. Forman, A.J.; Pass, D.A.; Connaughton, I.D. The characterisation and pathogenicity of porcine enteroviruses isolated in victoria. *Aust Vet J* **1982**, *58*, 136-142.
108. Mason, R.W. Porcine polioencephalomyelitis associated with a serological response to porcine enterovirus f.26 in tasmania. *Br Vet J* **1970**, *126*, xix-xx.
109. Cano-Gomez, C.; Palero, F.; Buitrago, M.D.; Garcia-Casado, M.A.; Fernandez-Pinero, J.; Fernandez-Pacheco, P.; Aguero, M.; Gomez-Tejedor, C.; Jimenez-Clavero, M.A. Analyzing the genetic diversity of teschoviruses in spanish pig populations using complete vp1 sequences. *Infect Genet Evol* **2011**, *11*, 2144-2150.
110. Prodělalová, J. The survey of porcine teschoviruses, sapeloviruses and enteroviruses b infecting domestic pigs and wild boars in the czech republic between 2005 and 2011. *Infection, Genetics and Evolution* **2012**, *12*, 1447-1451.
111. Prodelalova, J. The survey of porcine teschoviruses, sapeloviruses and enteroviruses b infecting domestic pigs and wild boars in the czech republic between 2005 and 2011. *Infect Genet Evol* **2012**, *12*, 1447-1451.
112. Cano-Gomez, C.; Garcia-Casado, M.A.; Soriguer, R.; Palero, F.; Jimenez-Clavero, M.A. Teschoviruses and sapeloviruses in faecal samples from wild boar in spain. *Vet Microbiol* **2013**, *165*, 115-122.
113. Boros, A.; Nemes, C.; Pankovics, P.; Kapusinszky, B.; Delwart, E.; Reuter, G. Porcine teschovirus in wild boars in hungary. *Arch Virol* **2012**, *157*, 1573-1578.
114. Kemeny, L.J. Isolation of transmissible gastroenteritis virus, pseudorabies virus, and porcine enterovirus from pharyngeal swabs taken from market-weight-swine. *Am J Vet Res* **1981**, *42*, 1987-1989.
115. Parker, B.N.; Wrathall, A.E.; Cartwright, S.F. Accidental introduction of porcine parvovirus and talfan virus into a group of minimal disease gilts and their effects on reproduction. *Br Vet J* **1981**, *137*, 262-267.
116. Hahnefeld, H.; Hahnefled, E.; Wittig, W. Talfan disease der schweine in deutschland. 1. Isolierung und charakterisierung von teschenvirus subtyp talfan bei saugferkeln im bezirk dresden. *Arch Exp Veterinarmed* **1965**, *19*, 185-218.
117. G Appel, P.S., VF Ohlinger, C Ewald. Enzephalomyopathie bei sauen und mastschweinen infolge einer infektion mit porzinem enterovirus (pev). *Tierärztl Umschau* **1995**, *50*, 326–336.
118. Zell, R.; Dauber, M.; Krumbholz, A.; Henke, A.; Birch-Hirschfeld, E.; Stelzner, A.; Prager, D.; Wurm, R. Porcine teschoviruses comprise at least eleven distinct serotypes: Molecular and evolutionary aspects. *J Virol* **2001**, *75*, 1620-1631.
119. Williams, C.R. *Focus on genome research*. Nova Biomedical Books: New York, 2004; p xiii, 424 p.
120. La Rosa, G.; Muscillo, M.; Di Grazia, A.; Fontana, S.; Iaconelli, M.; Tollis, M. Validation of rt-pcr assays for molecular characterization of porcine teschoviruses and enteroviruses. *J Vet Med B Infect Dis Vet Public Health* **2006**, *53*, 257-265.

121. Betts, A.O. Studies on enteroviruses of the pig. I. The recovery in tissue culture of two related strains of a swine polio-encephalomyelitis virus from the tonsils of 'normal' pigs. *Research in Veterinary Science* **1960**, *1*, 57-64.
122. Szent Ivanyi, T. Studies on swine enteroviruses. I. Isolation and serological grouping of strains. *Acta Microbiol Acad Sci Hung* **1963**, *10*, 125-128.
123. Morimoto, T.; Fujiwara, H.; Watanabe, M. Experimental infection of breast-fed piglets with porcine enteroviruses isolated in japan. *Natl Inst Anim Health Q (Tokyo)* **1965**, *5*, 1-12.
124. Wang, B.; Tian, Z.-J.; Gong, D.-Q.; Li, D.-Y.; Wang, Y.; Chen, J.-Z.; An, T.-Q.; Peng, J.-M.; Tong, G.-Z. Isolation of serotype 2 porcine teschovirus in china: Evidence of natural recombination. *Veterinary Microbiology* **2010**, *146*, 138-143.
125. Kasza, L. Swine polioencephalomyelitis viruses isolated from the brains and intestines of pigs. *Am J Vet Res* **1965**, *26*, 131-137.
126. Zell, R.; Krumbholz, A.; Henke, A.; Birch-Hirschfeld, E.; Stelzner, A.; Doherty, M.; Hoey, E.; Dauber, M.; Prager, D.; Wurm, R. Detection of porcine enteroviruses by nrt-pcr: Differentiation of cpe groups i-iii with specific primer sets. *J Virol Methods* **2000**, *88*.
127. Doherty, M.; Todd, D.; McFerran, N.; Hoey, E.M. Sequence analysis of a porcine enterovirus serotype 1 isolate: Relationships with other picornaviruses. *J Gen Virol* **1999**, *80 (Pt 8)*, 1929-1941.
128. Kaku, Y.; Sarai, A.; Murakami, Y. Genetic reclassification of porcine enteroviruses. *J Gen Virol* **2001**, *82*.
129. Jimenez-Clavero, M.A.; Fernandez, C.; Ortiz, J.A.; Pro, J.; Carbonell, G.; Tarazona, J.V.; Roblas, N.; Ley, V. Teschoviruses as indicators of porcine fecal contamination of surface water. *Appl Environ Microbiol* **2003**, *69*, 6311-6315.
130. Haack, S.K.; Duris, J.W.; Kolpin, D.W.; Fogarty, L.R.; Johnson, H.E.; Gibson, K.E.; Focazio, M.; Schwab, K.J.; Hubbard, L.E.; Foreman, W.T. Genes indicative of zoonotic and swine pathogens are persistent in stream water and sediment following a swine manure spill. *Appl Environ Microbiol* **2015**, *81*, 3430-3441.
131. Krumbholz, A.; Wurm, R.; Scheck, O.; Birch-Hirschfeld, E.; Egerer, R.; Henke, A.; Wutzler, P.; Zell, R. Detection of porcine teschoviruses and enteroviruses by lightcycler real-time pcr. *Journal of Virological Methods* **2003**, *113*, 51-63.
132. Chen, Q.; Gauger, P.C.; Stafne, M.R.; Thomas, J.T.; Madson, D.M.; Huang, H.; Zheng, Y.; Li, G.; Zhang, J. Pathogenesis comparison between the united states porcine epidemic diarrhoea virus prototype and s-indel-variant strains in conventional neonatal piglets. *J Gen Virol* **2016**, *97*, 1107-1121.
133. Chen, Q.; Li, G.; Stasko, J.; Thomas, J.T.; Stensland, W.R.; Pillatzki, A.E.; Gauger, P.C.; Schwartz, K.J.; Madson, D.; Yoon, K.J., *et al.* Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the united states. In *J clin microbiol*, 2014; Vol. 52, pp 234-243.
134. Li, H. Aligning sequence reads, clone sequences and assembly contigs with bwa-mem. **2013**.
135. Zhang, J.; Zheng, Y.; Xia, X.Q.; Chen, Q.; Bade, S.A.; Yoon, K.J.; Harmon, K.M.; Gauger, P.C.; Main, R.G.; Li, G. High-throughput whole genome sequencing of porcine reproductive and respiratory syndrome virus from cell culture materials and clinical

specimens using next-generation sequencing technology. *J Vet Diagn Invest* **2017**, *29*, 41-50.

136. Simpson, J.T.; Wong, K.; Jackman, S.D.; Schein, J.E.; Jones, S.J.; Birol, İ. Abyss: A parallel assembler for short read sequence data. *Genome Res* **2009**, *19*, 1117-1123.
137. Villanova, F.; Cui, S.; Ai, X.; Leal, E. Analysis of full-length genomes of porcine teschovirus (ptv) and the effect of purifying selection on phylogenetic trees. *Arch Virol* **2016**, *161*, 1199-1208.

APPEMDIX. LIST OF PTV-11 AND PTV-2 STRAINS

This list enumerates PTV-11 and PTV-2 strians identified for this thesis.

PTV-11 Strains

GenBank accession	Strain name	Isolation	Country	Sample	Reference
AF296096	Dresden	1965	Germany	CNS tissue	Zell *et al.*
AF296121	DS 1696/91	1991	Germany	Brain	Zell *et al.*
AY392536	RD 181/01	2001	Germany	N.A.	Zell *et al.*
AF296120	UKG 53/81	1981	U.K.	Feces	Zell *et al.*
AY392550	1008/88	1988	Germany	Spinal cord	Zell *et al.*
GQ293238	Vir 2374/01	2001	Germany	N.A.	Zell *et al.*
AM261026	Swine/IT/298/1996	1996	Italy	feces	La Rosa *et al.*
GQ502349	swine/IT/268641/2006	2006	Italy	feces	La Rosa *et al.*
GQ502348	swine/IT/268602/2006	2006	Italy	feces	La Rosa *et al.*
GQ502347	swine/IT/277121/2006	2006	Italy	feces	La Rosa *et al.*
GQ502346	swine/IT/147746/2007	2007	Italy	feces	La Rosa *et al.*
JF724040	CC82	2009	Spain	feces	Buitrago *et al.*
N.A.	OH264/2010	2012	Czech Republic	feces	Prodělalová *et al.*
N.A.	CAPM V-180	2012	Czech Republic	fetal tissues	Prodělalová *et al.*
AM261026	Swine/IT/298/1996	1996	Italy	feces	La rosa *et al.*

PTV-2 Strains

AF296087	T-80	1960	USA	Tonsils	Betts *et al.*
AF296110	Sek 49/99	1999	Germany	Organ pool	Zell *et al.*
AF296107	Vir 6711-12/83	1983	Germany	Brain	Zell *et al.*
AF296108	Vir 6793/83	1983	Germany	Brain	Auerbach *et al.*

GenBank accession	Strain name	Isolation	Country	Sample	Reference
AF296109	Vir 480/87	1987	Germany	Brain	Auerbach *et al.*
GQ293229	Vir 2018/87	1987	Germany	N.A.	Zell *et al.*
AY392534	DS 756/93	1983	Germany	Brain	Zell *et al.*
AY392533	DS 183/93	1983	Germany	N.A.	Zell *et al.*
AY392542	2-AK-III	1963	Hungary	Rectal swab	Szent-Ivanyi *et al.*
AY392541	12-PL	1963	Hungary	Rectal swab	Szent-Ivanyi *et al.*
AY392537	Stendal 2532	N.A.	Germany	N.A.	Zell *et al.*
AM261027	Swine/IT/320/1997	1997	Italy	Feces	La Rosa *et al.*
AB049554	SFK10	1990	Japan		Kaku *et al.*
GU446660	JF613	2009	China	Brain	Wang *et al.*
KX527849	ZJ16LX0401	2016	China	N.A.	Gu *et al.*
JF724046	CC90	2009	Spain	feces	Buitrago *et al.*
JF724045	CC89	2009	Spain	feces	Buitrago *et al.*
JF724044	CC87	2009	Spain	feces	Buitrago *et al.*
JF724043	CC86	2009	Spain	feces	Buitrago *et al.*
JF724042	CC85	2009	Spain	feces	Buitrago *et al.*
JF724038	CC80	2005	Spain	feces	Buitrago *et al.*
JF724036	CC78	2005	Spain	feces	Buitrago *et al.*
JF724035	CC77	2005	Spain	feces	Buitrago *et al.*
JF724034	CC76	2005	Spain	feces	Buitrago *et al.*
JF724033	CC75	2005	Spain	feces	Buitrago *et al.*
JF724032	CC74	2005	Spain	feces	Buitrago *et al.*
JF724029	CC71	2005	Spain	feces	Buitrago *et al.*
JF724028	CC70	2007	Spain	feces	Buitrago *et al.*
JF724026	CC67	2005	Spain	feces	Buitrago *et al.*
JF724023	CC60	2005	Spain	feces	Buitrago *et al.*
JF724022	CC59	2005	Spain	feces	Buitrago *et al.*
JF724019	CC49	2005	Spain	feces	Buitrago *et al.*

GenBank accession	Strain name	Isolation	Country	Sample	Reference
JF724016	CC45	2005	Spain	feces	Buitrago *et al.*
JF724015	CC44	2005	Spain	feces	Buitrago *et al.*
JF724014	CC43	2005	Spain	feces	Buitrago *et al.*
JF724009	CC37	2005	Spain	feces	Buitrago *et al.*
JF724005	CC29	2005	Spain	feces	Buitrago *et al.*
JF724004	CC28	2005	Spain	feces	Buitrago *et al.*
JF724002	CC26	2005	Spain	feces	Buitrago *et al.*
JF723999	CC22	2005	Spain	feces	Buitrago *et al.*
JF723998	CC21	2005	Spain	feces	Buitrago *et al.*
JF723996	CC19	2005	Spain	feces	Buitrago *et al.*
JF723995	CC18	2005	Spain	feces	Buitrago *et al.*
JF723988	CC5	2005	Spain	feces	Buitrago *et al.*
JF723987	CC4	2005	Spain	feces	Buitrago *et al.*
JF723986	CC3	2005	Spain	feces	Buitrago *et al.*
JF723985	CC2	2005	Spain	feces	Buitrago *et al.*
JF723984	CC1	2005	Spain	feces	Buitrago *et al.*
GQ502334	swine/IT/136514/2007	2007	Italy	feces	La Rosa *et al.*
GQ502333	swine/IT/88291/2007	2007	Italy	feces	La Rosa *et al.*
GQ502332	swine/IT/77378/2007	2007	Italy	feces	La Rosa *et al.*
GQ502331	swine/IT/70693/2007	2007	Italy	feces	La Rosa *et al.*
GQ502330	swine/IT/281874/2006	2006	Italy	feces	La Rosa *et al.*
GQ502329	swine/IT/280605/2006	2006	Italy	feces	La Rosa *et al.*
GQ502328	swine/IT/280327/2006	2006	Italy	feces	La Rosa *et al.*
GQ502326	swine/IT/277081/2006	2006	Italy	feces	La Rosa *et al.*
GQ502325	swine/IT/274566/2006	2006	Italy	feces	La Rosa *et al.*
GQ502324	swine/IT/273188/2006	2006	Italy	feces	La Rosa *et al.*
GQ502323	swine/IT/272217/2006	2006	Italy	feces	La Rosa *et al.*
GQ502322	swine/IT/270947/2006	2006	Italy	feces	La Rosa *et al.*

GenBank accession	Strain name	Isolation	Country	Sample	Reference
GQ502321	swine/IT/268624/2006	2006	Italy	feces	La Rosa *et al.*
GQ502320	swine/IT/266543/2006	2006	Italy	feces	La Rosa *et al.*
N.A.	Isolate 6335	1988	U.S.A.	Intestinal content	Janke *et al.*
N.A.	Isolate o3b	1966	U.S.A.	brain	Koestner *et al.*