RESEARCH REPORT
SWINE HEALTH

Title: Neuropathogenesis of a brain-derived *Porcine teschovirus* type 11 strain or a brain-derived *Porcine sapelovirus* strain in 3-week-old CDCD pigs-NPB #13-211

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Industry summary

Recently in the U.S., outbreaks of polioencephalomyelitis associated with porcine teschoviruses and/or sapeloviruses are being observed more frequently, affecting more pigs with wider age ranges and are lasting longer in affected herds than observed previously. A type 2 and 11 PTV appear to be emerging in addition to the type 1 PTV strains that have traditionally predominated. Economic losses in affected herds are significant and there are currently not methods to prevent or treat the disease. A recent extensive epizootic in Haiti with high morbidity and mortality may indicate the first episode of a highly virulent type 1 PTV in the Western hemisphere. This study will determine whether the isolated type 2 and 11 PTV are neuropathogenic and will help predict potential economic impact if they continue to emerge.

Keywords

Porcine enterovirus, porcine teschovirus serotype 2, porcine teschovirus serotype 11, porcine poliomyelitis

Introduction

A severe highly fatal polioencephalomyelitis affecting pigs of all ages was first reported in 1929 in Teschen, Czechoslovakia (Trefny 1930). Known as Teschen disease, the etiologic agent was a virulent neurotropic strain of type 1 porcine enterovirus that spread and caused devastating losses across Europe until it disappeared in the 1950s. Later, a milder form of the disease characterized by lower morbidity and mortality, less common mentation defects and predominantly posterior paresis and/or paralysis was reported in Talfan, Wales (Harding et al. 1957). The cause was determined to be a less virulent neurotropic strain of type 1 porcine enterovirus. Since then, this less virulent polioencephalomyelitic disease, known as Talfan disease, is reported in countries around the world in association with predominantly type 1, but less commonly types 2-6, 9 and 10 of porcine enterovirus (Alexandersen et al. 2012). Porcine enteroviruses were historically serotyped by virus-neutralization and 13 serotypes were described. They were further subdivided by physiochemical properties i.e. replication properties in various cell lines into three cytopathic effect (CPE) groups: group I (serotypes 1-7 and 11-13), group II (serotype 8) and group III (serotypes 9 and 10) (Zoletto 1965, Knowles et al. 1979). Subsequent analysis of genomic sequences confirmed the distinctness of the three groups and led to complete reclassification (Kaku et al. 2001, Zell et al. 2001) as follows: Porcine enterovirus group I serotypes 1-7, 11-13 are assigned to the new genus *Teschovirus* as porcine teschovirus (PTV) serotypes 1-10 and additional serotypes 11 – 13 are also described; porcine enterovirus group II, serotype 8, is

assigned to genus *Sapelovirus* as porcine sapelovirus (PSV); and porcine enterovirus group III, serotypes 9 and 10, remain as porcine enterovirus B (PEV-B).

In general, one or more strains of various types of PTV are endemic in commercial swine operations and most of growing and adult pigs have a serologic evidence of exposure to the virus. In one long-term longitudinal study spanning 26 months in a single swine herd, waves of infection by six different serotypes of PTV were demonstrated (Singh and Bohl 1972). The virus is known to be a resident in the pig gut and is commonly found in feces (Alexandersen et al. 2012). In contrast, Talfan-like nervous disease with polioencephalomyelitis is relatively uncommon and inconsistent in North America. Typically the problem in affected herds is sporadic and self-resolved. Recent evidence suggests that this may be changing. Beginning in 2009, a regional outbreak of teschoviral polioencephalomyelitis occurred in Haiti affecting swine of all ages with 60% morbidity and 40% mortality (Deng et al. 2012), marking the first report in the Western hemisphere of a highly virulent strain of PTV. The outbreak was caused by a type 1 PTV with 84% and 86% nucleotide identity by whole genomic sequencing with the index strains of Teschen and Talfan viruses, respectively. And, starting in December of 2012, Iowa State University's Veterinary Diagnostic Laboratory (ISUVDL) has received neurologic cases with posterior paralysis and sometimes encephalitic signs (abnormal mentation) 5 to 10 times more than previously from swine operations in wide geographic locations (Iowa, Illinois, Minnesota, North Carolina). These outbreaks have been in predominantly nursery-age pigs, but have also less commonly been in grower pigs or in adults. Outbreaks have also been apparently lasting longer and affecting more pigs than previously. Polioencephalitis and/or myelitis is/are consistently observed and PTV and/or PSV are detected by PCR in brain and/or spinal cord.

Sequencing of the PTV has frequently identified it as type 11. There have been no previous reports of type 11 PTV in cases of polioencephalomyelitis in North America. Likewise, porcine sapelovirus historically has not been associated with neurologic disease and is also frequently found in feces or intestine from clinically normal pigs. Hence, it is unexpected to detect PSV alone in brains and/or spinal cords from neurologic pigs. The significance of these findings is not clear, especially since fecal contamination during harvest of brains and spinal cords (often performed under field conditions by submitting veterinarians) may be the source of PCR-detected PTV and/or PSV. Therefore, the causative role of PTV11 and PSV in polioencephalomyelitis needs careful evaluation under experimental conditions. In addition, diagnosticians need a laboratory method such as in-situ hybridization or immunohistochemistry which can reliably visualize PTV or PSV within affected neurons in typical brain and spinal cord lesions, so that causality and not contamination can be interpreted from positive PCR test results.

Stated Objectives from original proposal

- Objective 1: Development of a novel porcine model to determine whether the isolated type 2 and 11 PTV are neuropathogenic and therefore capable of causing neurologic disease.
- Objective 2: Evaluation of histopathologic lesions within central nervous system and spinal cord, viral fecal titers, and fecal shedding in neonatal caesarian derived-colostrum deprived (CDC) pigs infected with porcine teschovirus type 2 and 11.

Materials and methods

Weaned pig study

Experimental design and housing

Eighteen, 5-weeks-old, cesarean derived and colostrum deprived pigs (CDCD) were purchased from a commercial source. Study animals were housed in three separated, environmentally controlled, rooms at ISU's Livestock Infectious Disease Isolation Facility (LIDIF). Animals were fed ad libitum with commercial porcine feed and rooms were kept at 80F for the duration of the study. CDCD piglets were randomly assigned to 3 treatment groups: GROUP 1 - negative control (n=4); GROUP 2 - PTV-2 (n=7); and GROUP 3 - PTV-11 (n=7). Enterovirus panel PCR was performed on fecal swabs in polls of five prior to challenge. After 72-hours of acclimation to the new facility pigs were sedated with intramuscular injection of TKX mixture (Telazol 500mg, Ketamine 250mg, Xylazine 250mg) at 4.4mg/kg prior to challenge. Animals from GROUP 1 were intravenously inoculated with 3ml of cell culture media; study animals from GROUP 2 and 3 were intravenously inoculated with 10^6 $TCID_{50}$ PTV-2 and 10^6 $TCID_{50}$ PTV-11, respectively. Inoculum was administrated using a 25 gauge butterfly catheter (Terumo™ Surflo™, Terumo Corporation; Shibuya, Tokyo, Japan) Pigs were clinically scored every other day (according to mention and spinal cord scoring system, Table 1) and serum, fecal swabs and nasal swabs were collected from all pigs on study days 0, 4, 8, 15, and 22 .

Table 1. Scoring system utilized to clinically evaluated pigs challenged with porcine teschovirus type 2 and 11.

	Mentation		**Spinal cord function**
0	Normal	**0**	Normal
1	Reduction in alertness	**1**	Mild incoordination of back legs
2	Marked depression and head tilt	**2**	Temporary ataxia of back legs
3	1 plus seizures or opisthotonus	**3**	Anterior and/or posterior ataxia +/- knuckling
4	2 plus events of seizures and opisthotonus	**4**	Posterior or quadraparesis or paralysis (recumbency)

Inocula:

PTV-2 and PTV-11 isolates were obtained from brain and/or spinal cords of pigs that were recently submitted to ISUVDL because of Talfan-like nervous disease with typical polioencephalomyelitis. These viruses have been purified in cell culture and confirmed to be free of other known swine viral pathogens, bovine viral diarrhea virus and mycoplasmas. Virus isolates were propagated and titrated in porcine kidney (PK-15) cells following the standard procedures established at ISUVDL. (ISU VDL, Procedure for Virus Isolation Test for Porcine Enterovirus and/or Porcine Teschovirus, Porcine Sapelovirus. Doc ID: 9.487/4)

Necropsy:

Total of eight pigs were necropsied at 15-days post challenge, two pigs from GROUP 1, two pigs from GROUP 2 and 4 pigs from GROUP 3. A pig from GROUP 2 was necropsied at 16-days post challenge due to progression of clinical signs of disease and in accordance with protocol-specific endpoints for humane euthanasia. Remaining piglets were necropsy at 22 days post challenge. Animals were clinically accessed prior to necropsy and videos were individually recorded from all necropsied animals. Macroscopic lesions were evaluated by a designated pathologist (PA and BA) and complete set of tissues including cerebrum; cerebellum; brainstem; cervical, thorax and lumbar sections of spinal cord; lung; tracheobronchial and mesenteric lymph nodes; heart; liver; spleen; kidney; sections of jejunum, duodenum and ileum; cecum; colon and sciatic nerve were collected. Half of samples were frozen at -80C and the other half were placed in 10% formalin for histopathologic examination. Three ~3mm segments of spinal cord from individual piglets were placed in RNAlater® for gene expression analysis and three sections were placed in glutaraldehyde (Glutaraldehyde 2.5% PBS PH 7.2; Poly Scientific R&D Corp. Bay Shore, NY, USA.) for electron microscopy.

Results

Study was just terminated one week ago therefore results presented here are preliminary at this point.

Neither teschovirus, enterovirus nor sapelovirus were detected by PCR on pool swabs prior to challenge. All study animals were sedated prior to challenge and no side effects were noted. Animals completely recovered from sedation within 45 minutes. Animals in GROUP 1 (control) remained healthy and developed no clinical signs. Three out of seven animals from GROUP 2 developed pasty to watery diarrhea at 7 days post inoculation (DPI) and mild incoordination of hind limbs was noted starting at 11 DPI. Watery to pasty diarrhea was observed in five out of seven pigs from GROUP 3, diarrhea started at 7 DPI and lasted for 3 days. At 10 DPI six out of seven animals showed mild to moderate depression. Starting at 11 DPI, clinical signs consistent with Talfan-like disease were observed in five out of seven animals. Posterior ataxia to paralysis was noted. Animals remained cognitively aware.

Histopathology: preliminary

The cervical, thorax and lumbar regions of the spinal cord were evaluated histologically. Spinal cord sections from all control pigs were unremarkable. All pigs from GROUP 2 had mild to severe lymphoplasmacytic myelitis with multifocal areas of gliosis, neuron degeneration and satelliosis in addition to small areas of hemorrhage (Figure 1). Lesions primarily affected the gray matter; however, blood vessels in the white matter are also occasionally surrounded by lymphocytes and plasma cells. Seven out of seven pigs challenged with teschovirus serotype 11 (GROUP 3) had moderate to severe spinal cord lesions, similar to lesions observed in animals from GROUP 2 (Figure 2A and 2B) . Interestingly, despite only three out of seven animals from GROUP 2 and six out of seven animals from GROUP 3 had notable clinical signs all animals had classic histopathologic lesions (severity of histologic lesions varied markedly between individual animals as well as section of spinal cord examined). Clinical signs are likely associated with the magnitude

of inflammatory reaction.

To our knowledge this is the first experimental study demonstrating the neuropathogenicity of teschovirus type 2 and 11. Affected animals developed classic clinical signs of posterior paralysis (video available) and consistent histopathologic lesions. Electron microscopy of the spinal cord, molecular diagnostic assays including porcine enterovirus, teschovirus and sapelovirus PCRs and virus sequencing, and gene expression are in progress.

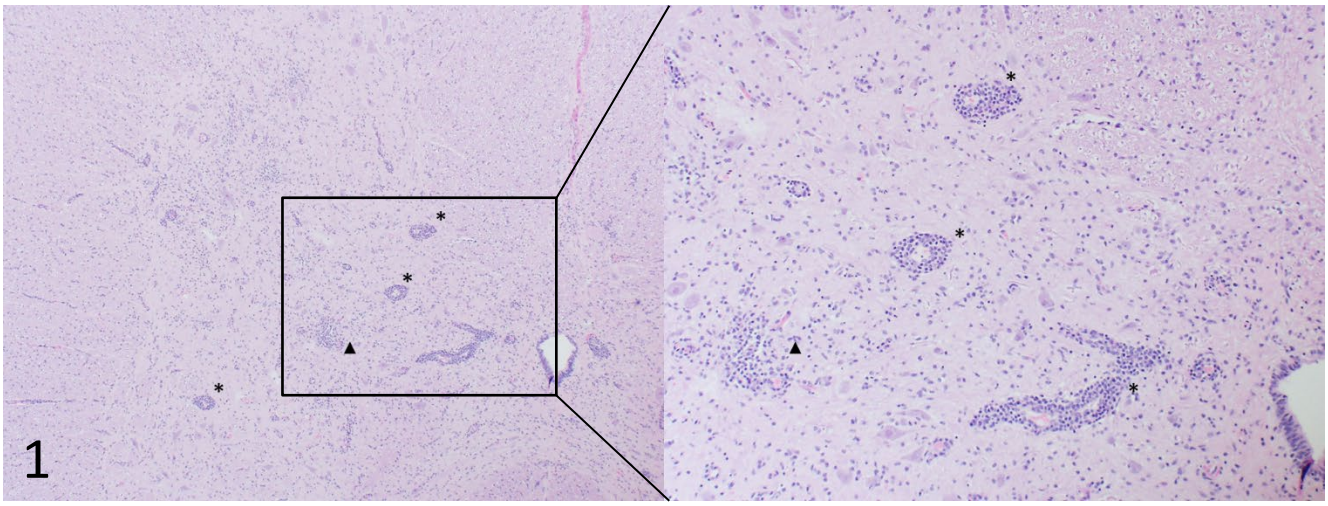

Figure 1. Cervical spinal cord from an animal challenged with teschovirus 2 and necropsied at 15 days-post inoculation. *Expansion of Virchow-Robin spaces by moderate to high numbers of lymphocytes and plasma cells. ▲Multifocal areas of gliosis.'

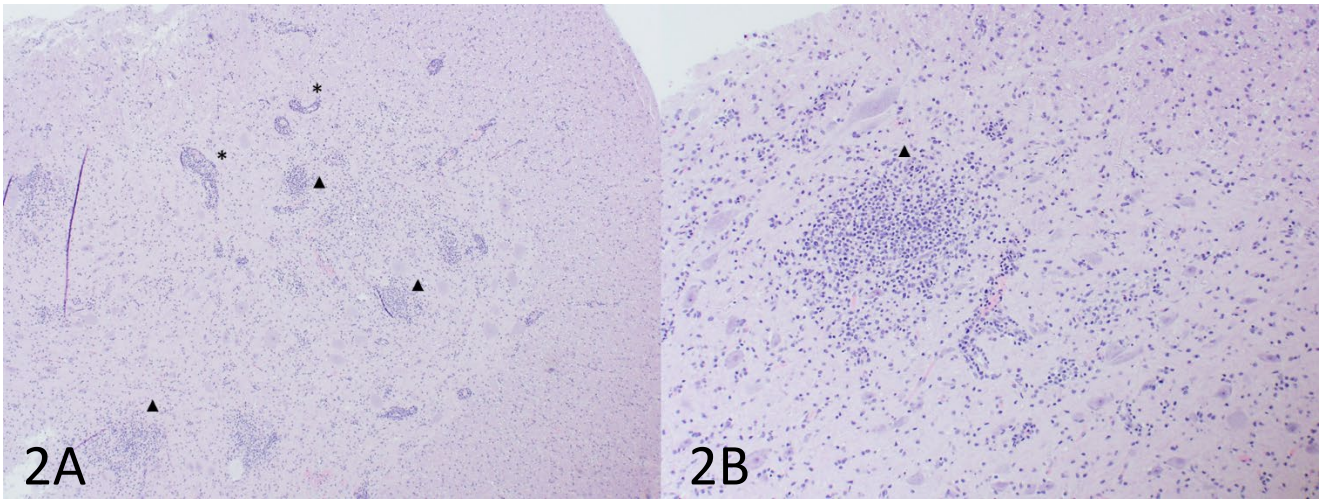

Figure 2A and 2B. Cervical spinal cord from an animal challenged with teschovirus 11 and necropsied at 22 days-post inoculation. *Expansion of Virchow-Robin spaces by moderate to high numbers of lymphocytes and plasma cells. ▲Multifocal areas of gliosis.

Modifications of project from original proposal

RE: NPB grant: Neuropathogenesis of a brain-derived Porcine teschovirus type 11 strain or a brain-derived Porcine sapelovirus strain in 3-week-old CDCD pigs

February 9, 2016

Dear Sir/Madam:

The studies funded by this grant were not performed on a timely basis, due to a variety reasons including Dr. Stevenson's illness. Due to my interest on this pathogen and with mutual consent of ISU VDPAM administration and involved faculty, I revived the protocol and organized a group of diagnosticians (Drs. Stevenson, Madson, Arruda, and Schwartz) and virologist (Dr. Zhang and Yoon) to complete this funded study. I have been a diagnostic pathologist at the Iowa State

Veterinary Diagnostic Laboratory (VDL) for the past 6 years and completed my pathology residence and PhD in 2014. I am currently an Assistant Professor at the ISU-VDL.

As proposed in the grant and stated in our interim report to NPB, we obtained pregnant gilts and aimed to raise CDCD piglets for this trial at an approved university facility (LIDIF). Unfortunately, pigs became infected with rotavirus which led to severe clinical signs, numerous deaths and ultimately to the humane euthanasia of all remaining piglets, hence were unable to meet the objectives of this research project.

We now plan to execute a shorter and more concise version of this experiment while still meeting a majority of the goals of the project by obtaining 4-week-old CDCD piglets from a commercial company. Using remaining funds from this grant as well as funding from another source, our plan is to purchase eighteen 4-week-old piglets (albeit CDCD piglets are expensive at $750 per piglet). We will execute the modified protocol and in doing so complete the Master's project of Dr. Franco M. Ferreyra who is a veterinarian on staff in the VDL and is familiar with execution and nuances of this project. The new IACUC for this modified attempt has been approved, and we intend to run the project in April of this year.

Basic experimental design as follows:

Groups	Inoculum	# pigs	Route of inoculation
1	10^6 TCID50 PTV-2	7	intravenous
2	10^6 TCID50 PTV-11	7	intravenous
3	Cell culture/control	4	intravenous

Thank you for your time, support and the opportunity to better understand Teschovirus in US swine.

Respectfully,

Thank you for funding this project. If there are any questions please feel free to contact me. Sincerely

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