Swine Health Information Center

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Diagnostic Assay Catalog

Part A: Polymerase Chain Reaction (PCR) Assays

Part B: Enzyme Linked Immunosorbent Assays (ELISAs) 20

Part A: Polymerase Chain Reaction (PCR) Assays

1. Porcine Cytomegalovirus Real-Time PCR Validation for Diagnostic Use (16-232)

Principal investigator	Susan Schommer (schommers@missouri.edu, tel. 573-882-4241)
Type of assay	PCR
Background information	The specific aim of this proposal was to develop and validate a real-time PCR assay for porcine cytomegalovirus (PCMV). The first objective was to sequence the polymerase gene of a United States PCMV. The second objective was to design and optimize the primer and probe for the real-time assay. Both published assays had mismatches to the United States PCMV strains. The third objective was to determine analytic sensitivity and specificity of the assay. The final objective was to compare antemortem sample testing to postmortem spleen, since this is a latent virus which is not always actively shedding.
Sample type	Blood, spleen, and oral fluid
Analytical sensitivity	5 copies/reaction Ct for PCR 1=35.51, Ct for PCR 2 = 34.45
Analytical specificity	100%
Diagnostic sensitivity	In animals with positive spleens: 44.8% (blood), 55.1% (oral fluid) were positive; when both blood and oral fluid were tested (and a positive result for either sample makes the animal positive), overall sensitivity increased.
Diagnostic specificity	No information; contact principal investigator.

Abstract: The specific aim of this proposal was to develop and validate a real-time PCR assay for porcine cytomegalovirus (PCMV). The first objective was to sequence the polymerase gene of a United States PCMV. This gene is the target for the two published real-time PCR PCMV assays but GenBank does not contain any sequences from US strains. Samples were collected from different sample types (spleen and blood) and different regions of the country (Missouri, Illinois, South Dakota, Minnesota, and Hawaii) to use for comparison to published primer and probe sequences.

The second objective was to design and optimize the primer and probe for the real-time assay. Both published assays had mismatches to the US PCMV strains. Modifications to the reverse primer and the probe of the genetically closest assay were tested and found to have no effect on detection (reverse primer modifications) or decrease detection (probe modification). In addition, the assay used was adapted to include an internal control for inhibition. Finally, since the levels of virus in antemortem samples were expected to be very low, a number of extraction methods and modifications were tested to see if sensitivity could be increased. While some methods improved the Ct value, none detected additional positives and all added significant processing time and cost. One direct lysis method shows promise for DNA pathogen detection in oral fluid as an inexpensive, low labor method but may not be appropriate for viruses with low concentrations such as PCMV.

The third objective was to determine analytic sensitivity and specificity of the assay. This assay does not cross react with any of the common porcine viruses tested and has a limit of detection of 5 copies/PCR reaction. Two copies could be detected 40% of the time. Results were similar when using spiked blood and oral fluid samples.

The final objective was to compare antemortem sample testing to postmortem spleen, since this is a latent virus which is not always actively shedding. In previous studies, virus levels in blood were shown to be below the limit of detection during latent infection; however using our protocol, 13/29 blood samples (44.8%) and 16/29 oral

fluid samples (55.1%) tested positive from animals with positive spleens. Overall accuracy was 50% for blood and 62% for oral fluid. When testing both blood and oral fluid, if an animal is considered positive when either sample is positive, accuracy improves to 70% and 69% of positives will be detected. While this is still a relatively low value, this includes animals of all ages; some of which may have been latent for a long period of time. Further analysis will include identifying and testing more negative animals and looking at the results as compared to age. Younger animals are more likely to be acutely infected and expected to have a higher rate of detection. This information would be useful for identifying what age groups to target when screening a herd for its status. Overall, it was determined that despite sequence differences, the previously published assay provided a sensitive real-time PCR test that can be used on both antemortem and tissue samples for detection of PCMV.

2. Development of Rapid Diagnostic Capability for Encephalomyocarditis Virus (16-233)

Principal investigator	Diego Diel (dgdiel@cornell.edu, tel. 607-253-4359)
Type of assay	PCR
Background information	The overall goal of this study was to develop and validate a probe-based real time PCR assay for rapid detection of encephalomyocarditis virus (EMCV). The first objective was to develop a real-time PCR assay for the detection of EMCV. The second objective was to evaluate and validate the newly developed assay for diagnostic applications.
Sample type	Oral, nasal, and rectal swabs and serum samples were collected from experimentally infected piglets and controls at days 0, 3, 7, 10, 14, and 22; heart, liver, spleen, tonsil, lymph node, intestine, and brain were collected from all animals at necropsy on day 22 pi for PCR testing.
Analytical sensitivity	$1.0x10^1$ genome copies/µl for control plasmid (3 trials; Ct=34.15, 33.38, and 34.53) $1.0x10^1$ $TCID_{50}$/ml equivalents for infected cell culture supernatant (3 trials; Ct=37.39, 37.96, 37.14)
Analytical specificity	100%
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	100%
Other	Heart was the best tissue sample for the detection of EMCV (RNA detected in heart samples from 8/10 piglets).

Abstract: Encephalomyocarditis virus (EMCV) is a positive sense single-stranded RNA virus that is capable of infecting several animal species including swine and humans. Rodents are thought to be the natural reservoirs of EMCV, spreading the virus to other susceptible animal species through contaminated feces or carcasses. EMCV causes acute myocarditis and mortality in young pigs or reproductive failure in sows. Mortality rates ranging from 20-100% have been reported in neonatal piglets. The EMCV genome has a unique coding region flanked by two untranslated regions (UTR). The 5' UTR is between 800-1200 nucleotides (nt) long and is highly conserved among different EMCV strains. The objective of this study was to develop and validate a probe-based real time PCR assay for rapid detection of encephalomyocarditis virus (EMCV). Given the high degree of conservation, the 5'UTR region of the EMCV genome was selected as the target sequence for the development of the real time assay.

To obtain clinical samples for validation of the assay, 5 week old piglets were divided into two groups and experimentally infected with EMCV as follows: control mock-infected group (DMEM; n=10) and EMCV strain Hawaii 1988-infected group (n=10; piglet source). Oral, nasal, rectal swabs and serum samples were collected

from the piglets on days 0, 3, 7, 10, 14, and 22 post-inoculation (pi). Heart, liver, spleen, tonsil, lymph node, intestine, and brain were collected from all animals at necropsy on day 22 pi for PCR testing. The analytical performance of the assay was assessed by evaluating its repeatability, analytical specificity and sensitivity, and diagnostic specificity and sensitivity. The limit of detection of the assay developed here was $1.0x10^1$ genome copies/µl for the control plasmid and $1.0x10^1$ $TCID_{50}$/ml equivalents for infected cell culture supernatant. The inclusive analytical specificity (100%) was assessed by testing the assay against five different EMCV strains, presenting between 82.6% - 89.7% nt identity amongst themselves. The exclusive specificity (100%) of the assay was assessed by testing clinical samples that were known to be positive for PRRSV, PCV2, PEDV, PDCoV, SVA, and porcine enterovirus 1, 3 and 7.

Heart was the best tissue sample for the detection of EMCV. After confirming the assay sensitivity and specificity, clinical samples collected from the animal experiment were tested. Samples from the group inoculated with EMCV strain Hawaii presented detectable EMCV RNA in the heart of eight out of the ten piglets. Additionally, EMCV RNA was also detected in oral, nasal and rectal swab samples. EMCV was not detected in any of the samples collected from control mock-inoculated group. This new assay has been well validated against multiple strains of EMCV and could be used with confidence by the VDLs.

3. Detection and Differentiation of Influenza Types A, B, C, and D in Swine (16-237)

Principal investigator	Feng Li (feng.li@uky.edu, tel. 605-688-6036)
Type of assay	PCR
Background information	The objective of this study was to develop a multiple RT-PCR assay that can detect and distinguish swine infections by four types of influenza viruses (IAV, IBV, ICV, and IDV).
Sample type	Lung, trachea, soft palate, nasal turbinates, and nasal washes (from ferrets and guinea pigs infected with IBV, ICV, and IDV); oral fluid samples from growers and breeders submitted to ADRDL.
Analytical sensitivity	10 copies/reaction
Analytical specificity	No information; contact principal investigator
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	No information; contact principal investigator
Other	21.1% of swine oral fluid field samples were positive for IAV.

Abstract: The objective of this study was to develop and validate a rapid diagnostic tool for regular surveillance of four types of swine influenza for the protection of swine health and also public health. There are four types of influenza viruses: A, B, C and D. In addition to influenza type A, swine are also infected by three other types of influenza viruses (B, C, and D). Increased outbreaks of newly identified influenza D in Europe and Asia with a novel bovine reservoir have raised a concern for both swine and human health. Individual real-time reverse transcription polymerase chain reaction (rtRT-PCR) tests for influenza A virus in swine (IAV-S) has been well established and already commercially available. Here, we reported on the development of a panel of multiplex rtRT-PCR assays that can detect and distinguish the conserved regions of all four types of swine influenza viruses. This panel of novel assays was designed to provide cost efficient testing to the producer and promote the continued surveillance for four types of influenza viruses. Following the experimental validation of the assay's specificity and sensitivity (10 copies of viral genome), a small-scale survey of field swine samples was conducted. Our results showed that among 90 oral fluid samples collected from Midwest swineherds, 21.11 % (19/90) were type A positive. Interestingly, none of these samples were tested positive for types B, C, and D.

Multiplex RT-PCR data obtained from this project are in good agreement with our previously reported serological data where we showed that less than 10% of surveyed pigs possessed antibodies against B or C or D, and that these antibodies were detected at low levels. Our findings suggest that non-A influenza infection of the U.S. swine population is very limited. In summary, the developed new multiplex RT-PCR assay for rapid differentiation of four types of swine influenza will play an important role in regular surveillance of swine populations for swine health, production, and the One Health Initiative.

4. Multiplex Real-Time RT-PCR Assay for Simultaneous Detection and Differentiation of Influenza C, D, and B Viruses (16-255)

Principal investigator	Xuming Liu
Current contact	Jianfa Bai (jbai@vet.ksu.edu, tel. 785-532-4332)
Type of assay	PCR
Background information	The overall goal of this proposal was to develop and validate a high-coverage, high-throughput, low-cost, and accurate multiplex real-time RT-PCR assay to simultaneously detect and differentiate ICV, IDV, and IBV. Specific objectives were to: 1) Design ICV, IDV, and IBV-specific primers and probes targeting the conserved viral gene regions; 2) Generate positive control standards for the development and optimization of real-time RT-PCR assays; 3) Optimize and validate uniplex real-time RT-PCR assays for specific detection of ICV, IDV, and IBV; and 4) Develop a multiplex real-time RT-PCR panel assay for simultaneous detection and differentiation of ICV, IDV and IBV.
Sample type	Nasal swab, oral fluid, serum, lung, blood, feces and stomached organ tissue homogenate
Analytical sensitivity	About 10 copies/reaction (range 6–12) Ct=36–38
Analytical specificity	100%
Diagnostic sensitivity	Accuracy IAV=98.2%, IDV=100% (no IBV and ICV clinical isolates from swine were available)
Diagnostic specificity	100%

Abstract: Influenza is one of the most common and highly contagious viral respiratory diseases caused by influenza A, B, C, and D viruses (IAV, IBV, ICV, and IDV), and causes significant economic losses every year. IAV is the most common pathogen, and previously was assumed to be the only influenza virus that could infect swine and cattle. Recent evidences demonstrate that IBV can infect swine and may affect cattle, ICV may also have the potential to infect swine and cattle, and the newly discovered IDV can infect both swine and cattle. Timely diagnosis of influenza viruses is important for prevention and intervention. IAV in human and animals has been well studied and various mature diagnostic assays are available, while there is a pressing need to develop new diagnostic assays especially multiplex assay for the rapid and accurate detection of the three under-studied and under-diagnosed influenza B, C, and D viruses together with IAV.

Real-time PCR is currently considered the most sensitive and practical approach for identification of influenza viruses and other pathogens in most diagnostic labs. Although a number of real-time PCR assays were published and currently used for diagnosis of influenza viruses, there are two major limitations associated with those assays: 1) Low coverage. *In silico* analyses indicated that the overall coverage rates for most of the previous real-time RT-PCR assays range from 40% to 60%, which are not capable of detecting the majority of current field strains and variants of ICV, IDV and IBV, and are not sufficient to meet the needs of influenza surveillance and

diagnosis. 2) Low multiplexing applications. All published influenza virus real-time PCR assays are uniplex or duplex assays that only target one or two of the four influenza viruses, and no multiplex real-time RT-PCR assay has been reported so far for simultaneous detection of any three or all four influenza A, B, C, and D viruses.

In this study, real-time PCR primers and probes are designed based on all complete and near-complete sequences of M segment of IBV and ICV, and PB1 segment of IDV from human and animals that are available in GenBank database. The IAV universal primers and probes are adapted from the USDA and CDC recommended real-time RT-PCR assays that are currently in use. An internal control targeting the host housekeeping gene (18S rRNA) is included to improve the assay accuracy by reducing false-negative results.

The *in silico* analysis indicated high strain coverages for IBV assay at 97.9% (6456/6593), ICV at 99.5% (185/186), and IDV at 100% (29/29). The plasmid DNA, in vitro transcribed RNA, and virus isolate RNA were used for analytical validation, and clinical samples were used for diagnostic validation. Results demonstrated that both the singular and multiplex assays are highly sensitive and specific in detection and differentiation of the target influenza viruses without cross-reactivity among influenza viruses nor to other common swine pathogens. The limit of detection was around 10 copies for each target. Diagnostic sensitivity analysis determined the reliable Ct cutoff values for IAV and ICV: Ct ≤37 are positives, Ct 37-39 are weak positives, Ct >39 are negatives; for IDV and IBV: Ct ≤36 are positives, Ct 36-38 are weak positives, and Ct > 38 are negatives.

In conclusion, the high-coverage (up to 98%-100%), high-sensitivity, high-multiplexing (5-plex), high-throughput, low-cost, specific and accurate one-step real-time RT-PCR assay developed in this study will greatly facilitate the convenient detection and differentiation of all four influenza A, B, C, and D viruses (plus an internal control) simultaneously in a single reaction for influenza diagnosis and surveillance purposes. The four ICV positive samples identified in this study are the first bovine ICV cases so far in the record. To our best knowledge, it is the first 5-plex real-time PCR assay that has been developed and validated so far for diagnostic applications.

5. Implementation of real-time RT-PCR to Detect Porcine Hepatitis E (HEV) (16-245)

Principal investigator	Doug Marthaler
Current contact	Rachel Palinski (rpalinski@vet.k-state.edu, tel. 785-532-5709 or 806-532-5650)
Type of assay	PCR
Background information	The objective of this study was *in silico* evaluation of Jothikumar's primers and probe, leading to a validation of real time RT-PCR to detect porcine hepatitis E.
Sample type	Feces, intestine, liver, oral fluid, serum
Analytical sensitivity	14.96 copies/reaction, Ct<36
Analytical specificity	100%
Diagnostic sensitivity	100%
Diagnostic specificity	100%

Abstract: Hepatitis E virus belongs to the Hepeviridae family and is a zoonotic agent, with pigs serving as a reservoir for the virus. The objective of the study was to evaluate and validate the previously published primer and probe set by Jothikumar et al. with an internal control targeting the 18S ribosomal RNA. The analytical sensitivity of the rRT-PCR was 14.96 copies per reaction, with a calculated PCR efficiency of 110.9. Analytical specificity was performed using 266 samples positive for 13 known porcine pathogens, and cross reactivity was not observed. A 100% diagnostic sensitivity and specificity was achieved by spiking different specimens with a known positive sample. A 0.96% occurrence of HepE was observed by testing 312 random diagnostic samples.

Even though the Jothikumar et al. primer and probe set was designed over 10 years ago, this set is acceptable to use in a diagnostic setting and has now been validated for high throughput diagnostic testing.

6. Development and Validation of a Real-Time PCR to Detect Porcine Torovirus (PToV) (16-247)

Principal investigator	Doug Marthaler
Current contact	Rachel Palinski (rpalinski@vet.k-state.edu, tel. 785-532-5709 or 806-532-5650)
Type of assay	PCR
Background information	The objective of this study was to develop and validate a real-time RT-PCR (rRT-PCR) to detect porcine torovirus.
Sample type	Feces, serum, oral fluid, intestine, tissue pool
Analytical sensitivity	877.6 copies/reaction
Analytical specificity	100%
Diagnostic sensitivity	106.34%
Diagnostic specificity	92.15%

Abstract: Belonging to the family Coronaviridae, toroviruses (ToVs) cause enteric disease in pigs, cattle, and pigs. A real-time RT-PCR (rRT-PCR) to detect porcine ToV (PToV) is lacking form the scientific literature. The objective of this study was to evaluate and validate a rRT-PCR to detect PToV for use in a veterinary diagnostic laboratory. The analytical sensitivity (limit of detection) was 877.6 copies of sDNA per rRT-PCR reaction. The PToV rRT-PCR efficiency was calculated at 100% and had a Coefficient of Variation ranged of 0.3-4.9% and 4.45-7.74% for intra-assay and inter-assay repeatability, respectively. The analytical specificity of the PToV rRT-PCR was 100%. The diagnostic sensitivity and specificity of the PToV rRT-PCR was 106.34% and 92.15%, respectively. The PToV rRT-PCR tests was validated for use in veterinary diagnostic laboratories.

7. Swine Disease Matrix Antigen Detection Research Pathogen #1: Nipah Virus (16-248)

Principal investigator	
Current contact	Bradley Pickering (bradley.pickering@inspection.gc.ca), tel. 204-789-7620)
Type of assay	PCR
Background information	The objective of this project was to improve current diagnostic methods for the detection of Nipah virus in swine. The specific aims of the project were to: 1) Determine susceptibility of north American swine to Nipah virus-Bangladesh (NiV-B); 2) Evaluate current Nipah virus-Malaysia (NiV-M) detection methods for ability to detect NiV-B-infected swine samples and modify if necessary; 3) Develop conventional RT-PCR assay targeting the polymerase (L) gene of both Nipah virus genotypes that is suitable for confirmatory sequencing; and 4) Transfer NiV detection assays to collaborating partner for further specificity testing and use with synthetic NiV RNA.
Sample type	Blood, nasal washes, rectal swabs, oral fluid, tissues
Analytical sensitivity	No information; contact principal investigator
Analytical specificity	NiV-B (fusion) and NiV (nucleoprotein) qRT-PCR assays: 100% NiV-B (fusion) had slightly enhanced specificity compared to NiV (nucleoprotein) with Ct values 0.4–0.5 cycles lower
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	No information; contact principal investigator

Abstract: Nipah virus (NiV) is classified as a category C agent with potential for bioterrorism and represents a major risk to human health and the livestock sector. While the original Malaysia epidemic followed transmission of NiV from swine to humans, subsequent outbreaks have occurred almost annually in Bangladesh through direct transmission from bats to humans with no confirmed involvement of pigs. As such, susceptibility of swine to NiV-Bangladesh is unknown, but considered highly probable. Given the potential risk to the swine sector and livestock producers, the objectives of this work were to determine susceptibility of North American commercial breed swine to Nipah Bangladesh, evaluate lower-risk group-sampling methods for virus detection in infected herds, assess the ability of currently utilized Nipah Malaysia-specific diagnostic tests to detect Nipah Bangladesh, and develop novel confirmatory assays able to detect and distinguish between the two genotypes.

The present work provides the first direct evidence that pigs are susceptible to Nipah Bangladesh, with virus shedding and infected tissues confirmed for all experimental animals despite absence of clinical signs. Collection and testing of multiple sample matrices showed oral chews to be a reliable group sampling method for NiV-B-infected animals, with positive RNA signals detectable throughout the course of experimental infection. Finally, qRT-PCR results suggest the Nipah Malaysia-based assay as a suitable diagnostic test for detection of both Nipah virus genotypes in infected pigs, while a newly developed NiV Bangladesh-specific assay can be subsequently utilized for differential genotyping.

8. Development and Validation of a Single-Tube, Triplex RT-PCR Assay for Differential Detection of Highly Virulent Chinese Strains of Pseudorabies Virus (16-250)

Principal investigator	Aruna Ambagala (aruna.ambagala@inspection.gc.ca, tel. 1-204-789-2013)
Type of assay	PCR
Background information	The main objective of this study was to develop and validate a single-tube triplex real-time RT-PCR assay for rapid and simultaneous detection and differentiation of highly pathogenic Genotype II PRV strains from Genotype I strains. The assay will contain an internal control for assessing DNA extraction efficiency and the presence of PCR inhibitors. Once validated, this assay will be made available to the US National Veterinary Diagnostic Laboratories (NVDL) and Canadian Animal Health Laboratories (CAHLN) for use in routine diagnostics and epidemiological studies.
Sample type	Whole blood, serum, nasal swabs, oral swabs, tissues
Analytical sensitivity	PRV classical and variant strains: 1 pfu (single infectious virus particle)
Analytical specificity	100%
Diagnostic sensitivity	Out of 246 experimentally infected pigs (oral and nasal swab samples), 131 samples were positive, 98 were negative, and 19 were suspicious (Ct >35.99)
Diagnostic specificity	100%

Abstract: Pseudorabies virus (PRV) causes Aujeszky's disease or pseudorabies, fatal encephalitis in newborn piglets, respiratory infection in growing and fattening pigs, and reproductive failures in pregnant sows. Like other herpesviruses, PRV establishes a lifelong latent infection in the peripheral nervous system followed by subsequent intermittent shedding of infectious virus. Pseudorabies has spread throughout the world, but Canada, Greenland and Australia are considered free of this disease.

In 2004, pseudorabies was eradicated from the commercial swine herds in the USA, but the virus remains in few localized feral swine populations. In late 2011, a newly emerged PRV variant with severe clinical signs and high mortality surfaced in pig herds in Northern China. Since then, this virus has spread across China causing severe

economic losses. If these highly pathogenic variants of PRV enter North America it could cause huge economic losses to the US and Canadian pork industries. Availability of a rapid, highly-sensitive, PCR-based diagnostic assay for differential detection of PRV variants is critical to prevent such losses. Here we describe development and validation of a single-tube triplex real-time-PCR assay for differential detection of variant strains of PRV. The assay targets the untranslated region between the US9 and US2 coding regions of the PRV genome and consists of two primers and two probes (one specific for classical strains and the other variant strains). This region is also part of the "gI/gE deletion" of Bartha K61 vaccine strain, enabling the assay to differentiate classical, variant, and vaccine strains. The assay contains an internal control to ensure adequate efficiency of nucleic acid extraction and to confirm the absence of PCR inhibitors in each sample.

The triplex assay did not show any cross reactivity with related herpesviruses or common porcine viruses, and was able to detect PRV classical and variant virus stocks diluted down to a single infectious virus particle. In reproducibility tests, the assay showed inter- and intra-assay coefficients of variations of 3.64 and 2.02 % respectively. The clinical sensitivity and specificity of the new single-tube triplex real-time PCR assay was validated using clinical samples (nasal and oral swabs, whole blood, serum, tissues) collected from pigs infected with either classical (Bristol) or variant (JS 2012 and HeN1) PRV strains and PRV-negative Canadian and US national herds. As expected the triplex assay did not detect viral DNA extracted from two commercial PRV differentiating infected from vaccinated animals (DIVA) vaccine strains Bartha K-61 and Bucharest. The assay was validated at the Kansas State University Veterinary Diagnostic Laboratory and at the National Veterinary Services Laboratory in Iowa.

In conclusion, in this study we have successfully developed and validated a highly sensitive and highly specific single-tube triplex real-time PCR assay for rapid detection and differentiation of newly emerged highly virulent PRV variant strains in China from the classical PRV strains. The assay will not detect pigs vaccinated with commercially available PRV DIVA vaccines and therefore can be used as a DIVA test. This assay will be made available to the National Veterinary Service Laboratories (NVSL) and other State and University Veterinary Diagnostic Laboratories in the USA, and Canadian Animal Health Laboratories (CAHLN) for routine diagnostics and epidemiological studies.

9. Development of a TaqMan Quantitative RT-PCR test for Porcine Parainfluenza Virus 1 (16-254)

Principal investigator	Yanhua Li
Current contact	Ying Fang (yingf@illinois.edu, tel. 217-300-5483)
Type of assay	PCR
Background information	The overall purpose of this study was to develop and validate a TaqMan qRT-PCR test for PPIV1 detection. Specific objectives were: 1) To design a set of primer and probe targeting conserved regions inside hemagglutinin gene of PPIV1; 2) To generate positive control standard for TaqMan qRT-PCR targeting PPIV1 hemagglutinin gene; 3) To optimize and validate the TaqMan qRT-PCR test.
Sample type	Serum, nasal swabs, oral fluid
Analytical sensitivity	10 copies/reaction
Analytical specificity	100%
Diagnostic sensitivity	100% (nasal swab and oral fluid)
Diagnostic specificity	100% (nasal swab and oral fluid)

Abstract: Porcine parainfluenza virus 1 (PPIV1) was first reported in the pig nasopharyngeal samples in Hong Kong in 2013. Recently, PPIV1 was determined to be widespread in US swine herds. However, no validated diagnostic assay is available for PPIV1 detection. In this report, a one-step real-time quantitative RT-PCR assay (qRT-PCR) targeting the viral hemagglutinin-neuraminidase (HN) gene of PPIV1 was developed and validated. No cross-reactivity was observed with nucleic acid prepared from common swine pathogens, including PRRSV, PCV2, IAV. The limit of detection was determined to be 10 copies per 20-µl reaction using in vitro transcribed HN RNA. The performance of this assay was further validated with 114 pig nasal swabs (56 known positive and 58 known negative samples for PPIV1) and 17 oral fluid samples (7 known positive and 12 known negative samples for PPIV1). The qRT-PCR results were consistent with RT-PCR and DNA sequencing of HN gene. This assay was further used to screen the diagnostic samples collected from 10 different farms. Among 310 nasal swab samples that we have tested, 201 samples from 8 farms were PPIV1 positive. Overall, this qRT-PCR assay demonstrates sufficient sensitivity and accuracy for detecting PPIV1 RNA. It will be a useful tool for the rapid diagnosis of PPIV1 infection and in aid of PPIV1 epidemiological surveillance.

10. Development of a Sensitive and Reliable Diagnostic Assay to Detect Atypical Porcine Pestivirus (APPV) in Swine (16-256)

Principal investigator	Lalitha Peddireddi (lalitha@vet.k-state.edu, tel. 785-532-5651)
Current contact	Lance Noll lwnoll@vet.k-state.edu
Type of assay	PCR
Background information	The overarching goal of this proposal was to develop a sensitive and reliable qRT-PCR assay and fully validate its use in accurately detection of APPV filed infections. Specific aims to achieve this objective were: 1) To design APPV-specific primers and probes; 2) To construct positive control standards; and 3) To optimize and validate the qRT-PCR assay.
Sample type	Serum, oral fluid, feces, nasal swab, and different tissue samples
Analytical sensitivity	~5 copies/reaction (NS3 assay), Ct=37 ~8 copies/reaction (NS5B assay), Ct=37
Analytical specificity	100%
Diagnostic sensitivity	89% (NS3 assay) 96% (NS5B) 100% (NS3 + NS5B)
Diagnostic specificity	100%

Abstract: Atypical porcine pestivirus (APPV), a highly divergent newly identified pestivirus, is reported as the etiologic agent for type AII congenital tremors in newborn piglets. Since the first report of this virus in the US swine herds in 2015, APPV has been reported in several countries around the world. Sequence analysis suggests high genetic variation (7-17%) among all currently known APPV strains reported from different parts of the world. Therefore, a sensitive and reliable PCR-based diagnostic test is critical for accurate detection of APPV. The main objective of this study is to develop a quantitative real-time RT-PCR (qRT-PCR) assay using all available and newly generated sequence information, for reliable detection of all currently known APPV strains. In this study, we have developed a triplex qRT-PCR assay, using all available sequences from GenBank as well as sequences newly generated from diagnostic samples. The triplex qRT-PCR assay included two APPV target regions (NS5B and NS3), to enhance assay coverage to detect highly divergent China strains reported recently, and host 18S rRNA target to serve as an internal control to monitor and eliminate any false negatives. Individual qRT-PCR assays for each target (NS3, NS5B and 18S rRNA) were optimized separately and then combined into a duplex

(NS3+18S and NS5B+18S) and triplex (NS3+ NS5B+18S) assays. Analytical and diagnostic validation of singleplex, duplex and triplex assays were performed using in vitro transcribed RNA, synthetic target sequences representing divergent China strains, APPV-positive and -negative samples from experimental infection studies and clinical samples submitted to KSVDL/ISUVDL. Diagnostic sensitivity of individual APPV assays was ~90% (NS3) and 97% (NS5B), respectively. When these assays are combined into a multiplex format, the diagnostic sensitivity of the multiplex assay increased to 100% with a limit of detection of less than 10 copies of APPV target sequences, which corresponds to a Ct of 37. No cross reactivity was observed for any of the assays with other common swine pathogens indicating high assay specificity. Retrospective screening of 1214 clinical samples, submitted to KSVDL over a period of 2 years, revealed ~15% prevalence of APPV in the US swine herds. APPV has been detected in samples from all age groups pigs, suggesting the possibility of APPV persistence in infected pigs. Of all the specimens tested, oral fluids appear to have higher viral loads (as indicated by low Ct) suggesting oral fluids as a better diagnostic specimen for APPV detection. Phylogenetic analysis of 35 NS5B partial sequences from APPV positive clinical samples, obtained from different states, revealed considerable sequence diversity (85.8% to 100% nucleotide identity) among APPV strains within the US. In addition, two full genome sequences obtained in this study exhibited significant sequence diversity (~12%) compared to the first isolate reported from the US in 2014. Notably, our qRT-PCR assay detected APPV in clinical samples submitted from Canada, which is the first report of APPV in this region. In summary, the triplex qRT-PCR assay developed in this study offers rapid and reliable detection of APPV in the US swine herds. Sequence variation among APPV filed strains reported in this study provides a basis for our understanding of genetic diversity and molecular epidemiology of APPV, currently being circulated within the US swine herds.

11. Detection and Differentiation of PCV3 from PCV2a, PCV2b, and the Highly Prevalent PCV2d Mutant Strains (16-257)

Principal investigator	Jianfa Bai (jbai@vet.ksu.edu, tel. 785-532-4332)
Type of assay	PCR
Background information	Objectives: 1) Develop a real-time PCR assay for the detection of PCV3 strains; 2) Re-evaluate and develop a real-time PCR assay for PCV2 strains with markedly improved diagnostic sensitivities; 3) Merge PCV3 and PCV2 assays and add an internal control to form a multiplex real-time PCR assay for the detection and differentiation of PCV3 and PCV2 strains; and 4) Sequence up to 50 PCV3 full genomes to further improve PCV3 detection sensitivity.
Sample type	Serum, blood, oral fluid, nasal swab, feces and stomached organ tissue homogenate
Analytical sensitivity	12 copies/reaction (PCV3 *cap* assay), Ct ≤37 10 copies/reaction (PCV2 assay), Ct ≤36
Analytical specificity	100%
Diagnostic sensitivity	100% (28/28) for samples positive by both current KSVDL PCV2 assay and new mqPCR (PCV2) Of 6 suspect positive samples (by new mqPCR), 4/6 were confirmed to be PCV2-positive by Sanger sequencing 52 selected PCV3 positives were confirmed by Sanger sequencing
Diagnostic specificity	Of 71 confirmed PCV2-negative samples, 64 tested negative (by new mqPCR) and 7 tested positive (Sanger sequencing confirmed that 4/7 were true positives).

Abstract: The newly identified porcine circovirus 3 (PCV3) is causing problems in swine similar to that caused by porcine circovirus 2 (PCV2). Yet the PCV3 genome shares little similarity to the PCV2 genome. Thus different molecular diagnostic assays need to be developed to detect and to differentiate the two viruses. The objectives of this study were to develop a multiplex real-time PCR assay that can detect and differentiate the majority of the field strains of PCV3 and PCV2. The other objective was to sequence about 50 PCV3 genomes in order to: 1) study the genetic diversity of the PCV3 strains; 2) perform phylogenetic analysis to identify if genetic mutations are effected by geographic locations (temporal effect was not considered as it is a new virus); and 3) to guide the potential modification to existing assays to ensure diagnostic coverage. Analyzing all available genome sequences in the PCR assay design may be the most important first step to ensure the diagnostic coverage of the assay. Some of the PCV2 assays published earlier now are only covering about ~50% or less of the field strains. It is necessary to re-analyze PCV2 sequences and redesign a new diagnostic assay. In this study we have analyzed 1907 available PCV2 full- or near-full genomes, and designed two sets of tests, each can detect 94.8% or 90.5% of the strains, and in combination can detect 98.9% of PCV2 strains including PCV2a, 2b, 2c, 2d and 2e strains. Our first PCV3 assay was designed based on the limited 32 genome sequences available at the time of design, and it covers all 32 sequences (100% coverage). However, when more sequences become available, both from the NCBI GenBank and from our own lab, the original design had mismatches to a few strains. To overcome this potential issue, a second set of tests was designed that in combination with the first design, can cover all 89 sequences with 100% coverage. Phylogenetic analysis of the 89 PCV3 full genomes indicated that the largest genetic diversity rate for PCV3 currently is 3.2%. Out of the 51 PCV3 genomes we sequenced, 37 were unique genomes, and most of them was clustered into different clusters together with published PCV3 genomes originated from different locations. As most of our home-sequenced samples were collected from the state of Kansas, our data indicated that the mutations in PCV3 strains in the past two years do not show a geographic distribution pattern, and they rather mutated randomly in the genome. The 3.2% mutation rate in the 2,000bp PCV3 genome represents 64 nucleotide mutations. These many nucleotide mutations in just two years indicated that the virus is changing, and thus continued monitoring the evolution of the virus may be necessary to trace the emerging strains and genotypes of the virus and their distributions. It is also important to modify molecular detection assays accordingly in order to keep assays up to date.

12. Development of PCR-Based Diagnostic Assays for Japanese Encephalitis Virus Infection in Pigs (16-258)

Principal investigator	Dana Vanlandingham (dlvanlan@vet.k-state.edu, tel. 785-532-1300)
Type of assay	PCR
Background information	The overall objective of this study was to evaluate real-time RT-PCR assays targeting consensus regions of JEV to detect JEV in tissue culture, and serum, brain, and oral fluid samples in pigs. Objective 1: Development and evaluation of the JEV diagnostic assay using tissue culture fluid (sensitivity analysis), swine serum samples spiked with JEV and other flaviviruses (specificity analysis), and swine serum samples and brain tissue samples collected from the acute phase of the disease in a previously performed challenge study conducted in the summer of 2016. Objective 2: Evaluate the developed assay using swine oral fluids collected from the acute and convalescent phases of the disease from a planned 2017 experiment with JEV infected pigs.
Sample type	Serum, lymphoid, and central nervous system (CNS) tissues, oral fluid
Analytical sensitivity	Biological samples: 15.7 $TCID_{50}$/ml (assay 1, targeting thumb motif of the RNA-dependent RNA

	polymerase domain in the nonstructural protein 5 [NS5] gene0 36.4 $TCID_{50}$/ml (assay 2, targeting the sequences of the 5' dumbbell region and the 3' conserved sequence 1 in the 3' UTR of the JEV genome) 43.3 $TCID_{50}$/ml (assay 3, based on the pan-flavivirus FU4-CFD2 primers) Synthetic cDNA: 12.5 cDNA copies/ml (assay 1) 49.4 cDNA copies/ml (assay 2) 47.7 cDNA copies/ml (assay 3)
Analytical specificity	100%
Diagnostic sensitivity	Assay 1 successfully detected JEV in lymphoid and CNS tissues collected from intravenously challenged domestic pigs (see paper for viral loads) Assays 1 and 2 successfully detected JEV in oral fluid
Diagnostic specificity	No information; contact principal investigator

Abstract: Background. Japanese encephalitis virus (JEV) is a zoonotic flavivirus endemic in the Asia Pacific region. Maintenance cycles of JEV rely on the transmission vectored by competent mosquitoes among viremic avian and swine species. Although domestic pigs play a significant role in the ecology of JEV, there have been very few studies characterizing the tropism and disease pathogenesis of JEV in domestic pigs. In addition to the lack of our knowledge in the infection process of JEV, even fewer studies have been conducted to establish diagnostic methods for swine species infected with JEV. The lack of standardized and optimized diagnostic methods precludes the further analyses of data from different surveillance programs in endemic countries and the establishment of standardized procedures to monitor the dispersal of JEV to other regions. In this study, three JEV-specific real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assays were evaluated for the detection of JEV genome in serum, infected tissues and oral fluid of experimentally challenged pigs to establish an optimized procedure for the quantitative detection of JEV in infected pigs. The use of domestic pigs and miniature feral pigs from the United States is also expected to improve the basis of our knowledge in the susceptibility of North American pigs to JEV and the disease pathogenesis and tissue tropism in infected pigs.

Materials and methods. Because of the conserved sequences in the nonstructural protein 5 (NS5) gene and the 3' untranslated region (UTR) among all JEV strains and related flaviviruses, three real-time RT-PCR assays targeting the NS5 gene and 3' UTR were selected for the evaluation of diagnostic assays for JEV infection in swine species. Sensitivity of each assay was determined by constructing standard curves using serially diluted viral RNA extract from tissue culture medium and synthetic RNA genomes and complementary DNA (cDNA) fragments. Cross-reactivity of the three assays with two other medically important flaviviruses under the JEV serocomplex, West Nile virus (WNV) and St. Louis encephalitis virus (SLEV), was tested using synthetic viral genome. Viral RNA extracts from the serum, central nervous system (CNS) tissues, and oral fluid collected from experimentally challenged 3-week-old piglets were analyze by all three real-time RT-PCR assays to identify the optimized protocol for the detection of JEV in different samples derived from infected animals.

Results. Whilst the three assays were designed to target the neighboring regions of the 3' end of the viral genomes, a difference in the limit of detection among the three assays was observed. The primers and probe designed to anneal to the sequences in the thumb motif of the RNA-dependent RNA polymerase domain in the NS5 gene led to the highest sensitivity in the detection of viral genome in the viral RNA extract derived from tissue culture medium and synthetic cDNA fragments. Similarly, the assay also showed the highest sensitivity against all samples collected from infected animals including serum, CNS, lymphoid tissues, and oral fluid.

Major finding. The results of our study identified a sensitive and specific molecular detection method that can be used in the diagnosis of JEV in the event of its introduction. The assay can be used for the detection of JEV

genomes in various infected tissues. The method also allows the detection of JEV genomes in the oral fluid collected from infected animals using the rope-based collection method described in this study. The new procedure is less invasive and provides a significantly higher throughput than the detection of JEV in serum samples collected from viremic animals. The application to the JEV surveillance programs based on sentinel pigs in the endemic region has the potential of increasing the throughput of existing detection methods and reducing the technical requirement for the collection of diagnostic samples.

Future directions. The detection of JEV in various infectious samples has demonstrated the feasibility of using molecular detection techniques as diagnostic methods for JEV infections in pigs. The development of the oral fluid sampling technique provides the method for rapid sampling in response to potential outbreaks. Studies on the oral shedding of individual infected pigs will determine the potential of its further application to veterinary diagnosis.

13. Development of a Dry Room Temperature-Stable Real-Time RT-PCR Assay for the Detection of Porcine Hemagglutinating Encephalomyelitis Virus (16-259)

Principal investigator	Luis Gimenez-Lirola (luisggl@iastate.edu, tel. 515-294-7025)
Type of assay	PCR
Background information	The purpose of this study was to: 1) Develop of a dry room temperature-stable real-time RT-PCR assay for the specific detection of PHEV; and 2) Describe and compare the patterns of PHEV viremia (individual pig serum) and virus shedding (pen-based feces and oral fluids) in pigs experimentally inoculated with PHEV. This was a collaborative project between ISU-VDL and Tetracore, Inc. The ultimate goal is the development of a standardized PHEV rRT-PCR kit easy implementation in all U.S. VDLs.
Sample type	Serum, feces, oral fluid
Analytical sensitivity	No information; contact principal investigator
Analytical specificity	No information; contact principal investigator
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	No information; contact principal investigator
Other	Virus shedding of greatest duration detected in oral fluid compared to feces (1–28 dpi vs. 1–10 dpi, respectively); viremia not detected.

Abstract: Porcine hemagglutinating encephalomyelitis virus (PHEV) is the only known neurotropic coronavirus affecting pigs, being a potential threat to high health gilts herds. PHEV can infect naïve pigs of any age but clinical disease is age-dependent. In growing pigs and adults, PHEV infection is subclinical, but acute outbreaks of vomiting and wasting syndrome, and encephalomyelitis may be seen in neonatal pigs born form naïve sows, with mortality rates reaching 100%. To date, there was not diagnostic tools available among U.S. Veterinary Diagnostic Laboratories (VDLs). Moreover, there are not previous information describing kinetic of PHEV shedding (i.e., duration and pattern of viral shedding, specimen more suitable for testing) in the field nor under experimental conditions. The implementation of PCR-based methods will help to identify and subsequently isolate animals who are actively shedding the virus. Therefore, the goal of this collaborative project between Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) and Tetracore Inc. funded by SHIC was to develop and optimize a standardized a PHEV real-time RT-PCR kit of easy implementation in all U.S. VDLs. To achieve this objective, we experimentally inoculated twelve 7-week-old pigs (12 pigs, 6 pens, 2 pigs per pen). Pen-based oral fluid and fecal specimens were collected daily from day post-inoculation (DPI) -7 through 42.

Serum samples were collected at DPI -7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42. We also described and compare the patterns of PHEV shedding in pen-based feces and oral fluid specimens collected over the course of the infection. Virus shedding was consistently detected by real-time RT-PCR assay in pen oral fluids (DPI 1-28) and feces (DPI 1-10). Viremia was not detected throughout the observation period. Study results indicated that oral fluids are a suitable specimen for routine PHEV diagnosis and surveillance. This is consistent with the fact that oral fluids have become the preferred sample type used in monitoring for pig diseases.

14. Validation of a Real-Time Reverse Transcription PCR Assay for Detection of Porcine Sapelovirus (PSV) (16-269)

Principal investigator	Karen Harmon (kharmon@iastate.edu, tel. 515-294-1950)
Type of assay	PCR
Background information	Objectives: 1) Evaluate specificity of newly designed (ISU) and published (Chen) qPCR assays for detection of PSV; 2) Determine Limit of Detection of ISU and Chen assays; 3) Compare performance (positive/negative status and Ct values) of ISU and Chen assays with each other and nested gel-based PCR (Zell et al.); and 4) Sequence positive and negative samples for verification of specificity.
Sample type	Spinal cord and brain homogenates, serum, feces, and oral fluid
Analytical sensitivity	11.2 copies/µl (ISU) 3.18 copies/µl (Chen) The Chen assay was more sensitive than the ISU assay, generally producing earlier Cts, having a lower LOD, and having a higher rate of detection than the ISU assay.
Analytical specificity	100% (ISU) 100% (Chen)
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	No information; contact principal investigator

Abstract: Porcine sapelovirus (PSV), a member of the family Picornaviridae, is one of numerous biological agents which can be associated with neurological signs in swine. While detection of this agent is not necessarily a conclusive diagnosis, it is a critical component for diagnosis as well as for further studying the role of this agent in clinical disease. This project addressed assessment of two real-time reverse transcription PCR (qPCR) assays for the detection of PSV. One assay (Chen et al.) had been previously published, and the other assay (ISU) was newly designed based on sequence information available in GenBank. Both qPCR assays were run on 231 samples submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). Results from both assays were compared with each other in addition to those from a published nested gel-based PCR assay (Zell et al.). A subset of positive and negative samples were further subjected to sequencing of a portion of the VP1 gene, which has traditionally been the target of choice for identification of this family of viruses, to verify PSV status of the samples. In addition, a dilution series of target-specific RNA generated by in vitro transcription was tested to determine the limit of detection of the two qPCR assays. Both the Chen and ISU qPCR assays generated positive results for both PSV-8b and PSV 8C. Neither qPCR assay yielded positive results for any of the following agents: PTV (PTV-1, PTV-2, PTV-4, PTV-5, PTV-7, PTV-11 or PTV-12) PRRSV (including one identified from a neurologic case at ISUVDL), Congenital Tremor Pestivirus, PCV2, PEDV, TGEV, PDCoV, porcine hemagglutinating encephalomyelitis virus (PHEV) and encephalomyocarditis virus (EMC). We were unable to obtain an isolate or known positive sample for porcine enterovirus (PEV). However, primers and probes were determined to be in genomic regions which do not share similarity with PEV, as determined by both BLAST searches and by examining alignments containing both PSV and PEV sequences. The Limit of Detection (LOD) for the ISU and

Chen assays were determined to be 11.2 and 3.18 copies/µl, respectively. VP1 sequencing confirmed the presence of PSV in 54 samples that tested positive for PSV, but not in 98 that tested negative for PSV, corroborating the specificity of both qPCR assays. The Chen assay was more sensitive than the ISU assay, generally producing earlier Cts, having a lower LOD, and having a higher rate of detection than the ISU assay. Based on this study, our recommendation would be to use the Chen PCR assay for diagnostic testing of PSV.

15. Validation of a Real-Time Reverse Transcription PCR Assay for Detection of Porcine Teschovirus (PTV) (16-270)

Principal investigator	Karen Harmon (kharmon@iastate.edu, tel. 515-294-1950)
Type of assay	PCR
Background information	Objectives: 1) Evaluate specificity of newly designed (ISU) and published (Jimenez) qPCR assays for detection of PTV; 2) Determine Limit of Detection of ISU and Jimenez assays; 3) Compare performance (positive/negative status and Ct values) of ISU and Jimenez assays with each other and nested gel-based PCR (Zell et al); and 4) Sequence positive and negative samples for verification of specificity.
Sample type	Spinal cord and brain homogenates, serum, feces, and oral fluid
Analytical sensitivity	3.0 copies/µl (ISU) 1.97 copies/µl (Jimenez) The Jimenez assay was more sensitive than the ISU assay, generally producing earlier Cts, having a lower LOD, and having a higher rate of detection than the ISU assay.
Analytical specificity	100% (both)
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	No information; contact principal investigator

Abstract: Porcine teschovirus (PTV), a member of the family Picornaviridae, is one of numerous biological agents which can be associated with neurological signs in swine. While detection of this agent is not necessarily a conclusive diagnosis, it is a critical component for diagnosis as well as for further studying the role of this agent in clinical disease. This project addressed assessment of two real-time reverse transcription PCR (qPCR) assays for the detection of PTV. One assay (Jimenez et el.) had been previously published, and the other was newly designed based on sequence information available in GenBank. Both qPCR assays were run on 256 samples submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL). Results from both assays were compared with each other in addition to those from a published nested gel-based PCR assay (Zell et al.). A subset of positive and negative samples were further subjected to sequencing of a portion of the VP1 gene, which has traditionally been the target of choice for identification of this family of viruses, to verify PTV status of the samples. In addition, a dilution series of target-specific RNA generated by in vitro transcription was tested to determine the limit of detection of the two qPCR assays. Both the Jimenez and ISU qPCR assays generated positive results for PTV-1, PTV-2, PTV-4, PTV-5, PTV-7, PTV-11 and PTV-12 but not for either PSV-8b or PSV 8C. Neither qPCR assay yielded positive results for any of the following agents: PRRSV (including one identified from a neurologic case at ISUVDL), Congenital Tremor Pestivirus, PCV2, PEDV, TGEV, PDCoV, porcine hemagglutinating encephalomyelitis virus (PHEV) and encephalomyocarditis virus (EMC). We were unable to obtain an isolate or known positive sample for porcine enterovirus (PEV). However, primers and probes were determined to be in genomic regions which do not share similarity with PEV, as determined by both BLAST searches and by examining alignments containing both PTV and PEV sequences. The Limit of Detection for the ISU and Jimenez assays were determined to be 3.0 and 1.97 copies/µl, respectively. VP1 sequencing confirmed the presence of

PTV in 43 samples that tested positive for PTV, but not in 86 samples that tested negative for PTV, corroborating the specificity of both qPCR assays. The Jimenez assay was more sensitive than the ISU assay, generally producing earlier Cts, having a lower LOD, and having a higher rate of detection than the ISU assay. Based on this study, our recommendation would be to use the Jimenez assay for diagnostic testing of PTV.

16. Development and Evaluation of a Real-Time RT-PCR and a Field-Deployable RT-Insulated Isothermal PCR for the Detection of Seneca Valley Virus (16-271)

Principal investigator	Jianqiang Zhang (jqzhang@iastate.edu, tel. 515-294-8024)
Type of assay	PCR
Background information	Development and validation of a real-time RT-PCR (rRT-PCR) and a field-deployable RT-insulated isothermal PCR (RT-iiPCR) for the detection of Seneca Valley virus.
Sample type	Vesicular swabs, tonsil swabs, oral fluids, serum, and fecal swabs
Analytical sensitivity	3.5 copies/reaction (rRT-PCR) 7 copies/reaction (RT-iiPCR)
Analytical specificity	100% (rRT-PCR) 100% (RT-iiPCR)
Diagnostic sensitivity and specificity	Sensitivity and specificity varied according to extraction methods and instruments: sensitivity=97.26%–100%; specificity=98.08%–100%; accuracy=98.4%–99.20%.

Abstract: Seneca Valley virus (SVV) has emerged in multiple countries in recent years and SVV infection causes vesicular lesions clinically indistinguishable from those caused by other vesicular disease viruses, such as the highly regulated foot-and-mouth disease virus, swine vesicular disease virus, vesicular stomatitis virus, and vesicular exanthema of swine virus. Sensitive and specific RT-PCR assays for the SVV detection is necessary for differential diagnosis. Real-time RT-PCR (rRT-PCR) is a sensitive and reliable assay for the detection of many RNA viruses. The insulated isothermal PCR (iiPCR) on a portable POCKIT™ device is user friendly for on-site pathogen detection. In the present study, a SVV rRT-PCR targeting the conserved 5' untranslated region and a SVV RT-iiPCR targeting the conserved 3D gene were developed and validated. Neither the SVV rRT-PCR nor the SVV RT-iiPCR cross-reacted with any of the vesicular disease viruses that included 20 FMDV strains, 2 SVDV strains, 6 VSV strains, and 2 VESV strains. The SVV rRT-PCR and RT-iiPCR also did not cross-react with the 4 strains of classical swine fever virus and any of other 15 common swine viruses. Limit of detection (LOD) based on in vitro transcribed RNA was 3.5 genomic copies for the rRT-PCR and 7 genomic copies per reaction for the RT-iiPCR. The LOD of SVV rRT-PCR and RT-iiPCR in the unit of $TCID_{50}$/ml was also determined using serially diluted US historical SVV isolate (ATCC SVV001) and US contemporary SVV isolate (USA/SD41901/2015) and both assays had comparable analytical sensitivities. For diagnostic performance evaluation, 125 swine samples (12 vesicular swab, 30 tonsil swab, 25 oral fluid, 28 serum, and 30 fecal swab samples) collected from various states within the USA in 2015-2016 were tested. For clinical samples testing, two approaches were conducted. First, nucleic acids prepared by the MagMAX™ Pathogen RNA/DNA Kit were tested by both PCR assays. Second, the same sample set was tested by the RT-iiPCR in the POCKIT™ combo system (taco™ mini/RT-iiPCR) and by the MagMAX™/rRT-PCR system, respectively. Among the 125 MagMAX™ extracts, one was negative by the rRT-PCR but positive by the RT-iiPCR, resulting in an agreement of 99.20% (124/125; 95% CI: 96.59-100%, κ=0.98). Among 125 samples, two were positive by the MagMAX™/rRT-PCR system but negative by the taco™ mini/RT-iiPCR system, resulting in a 98.40% agreement (123/125; 95% CI: 95.39-100%, κ=0.97). The two samples with discrepancy results by the two PCR assays had high C_T values (35-36). In conclusion, the SVV rRT-PCR and RT-

iiPCR assays are very sensitive and specific and have comparable diagnostic performances for SVV RNA detection from clinical samples. The SVV rRT-PCR assay can be adopted for SVV detection in laboratories. The SVV RT-iiPCR in a simple field-deployable system could serve as a tool to help swine vesicular disease diagnosis at points of need.

17. Detection and Differentiation of Seneca Valley Virus (SVV) from Foot-and-Mouth Disease Virus (FMDV) (16-272)

Principal investigator	Jianfa Bai (jbai@vet.ksu.edu, tel. 785-532-4332)
Type of assay	PCR
Background information	Objective 1. Re-evaluation of KSVDL SVV assay and rebuilding positive control plasmid if needed; Objective 2. Incorporate a general FMDV assay and an internal control into the current or modified assay to form a triplex real-time PCR assay for SVV and FMDV detection. An endogenous or spike-in internal control will be used to ensure RNA extraction efficiency and monitor potential PCR inhibitions. The newly formed duplex PCR will be compared with singular PCRs to ensure that the analytical sensitivity is not impaired by multiplexing; Objective 3. Diagnostic validation with pig nasal and fecal samples; Objective 4. Diagnostic validation with 1) FMDV strains, and 2) diagnostic/field samples positive to different combinations of common swine pathogens.
Sample type	Serum or blood, oral fluid, nasal swab, feces, swab lesion and stomached organ tissue homogenate
Analytical sensitivity	0.8 $TCID_{50}$/ml, Ct=37 (SVV-1, multiplex assay) 2.5–18.0 $TCID_{50}$/ml, Ct=36–40 (FMDV, seven serotypes, multiplex assay)
Analytical specificity	100%
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	No information; contact principal investigator

Abstract: This study was aimed to develop a multiplex real-time PCR assay for the detection and differentiation of Seneca Valley virus 1 (SVV-1) and foot and mouth disease virus (FMDV). The SVV-1 virus induces clinical sign that is indistinguishable from the disease caused by FMDV. We recently developed an updated version of our previous SVV-1 real-time (r) reverse-transcription (RT) PCR (rRT-PCR) assay to accommodate the detection of the increasing genetic diversity of SVV-1 strains. One of the OIE recommended FMDV rRT-PCR assays targeting the highly conserved 3D gene has been widely used. An *in silico* analysis of the primer/probe indicated that 59% of the strains analyzed has a perfect sequence match to the primer/probe sequences; thus the predicted diagnostic coverage is 59%. However, the coverage is increased to 88% if up to 2-bp mismatches on any oligo is allowed. Standard curves using viral strains of all seven FMDV serotypes were generated. The PCR amplification efficiencies of the assay ranged from 51% to 66% across the seven serotypes. We have developed a new FMDV assay that has a strain coverage of 84-94%, from perfect match to up to 2-bp mismatches to any primers and probe. Standard curves of our assay on the seven FMDV serotypes revealed 84-98% PCR amplification efficiencies. This new FMDV assay was subsequently multiplexed with the updated SVV-1 assay and an 18S rRNA gene as internal control. The internal control amplifies the target gene in multiple species including swine, bovine, ovine, caprine and cervine. Standard curves indicated that correlation coefficients (R2 values) of the assay were all greater than 0.99, and PCR amplification efficiencies were 89-98%. Limit of detection (LOD) for this multiplex rRT-PCR assay for SVV-1 was Ct 37 (0.8 TCID50/mL); and LODs for the seven FMDV serotypes were Ct 36-40 (2.5-18.0 TCID50/mL), and there was no loss of sensitivity by multiplexing. This multiplex rRT-PCR assay

is able to detect and differentiate SVV-1 from FMDV infection in a single PCR reaction, and has noticeable improvement in both analytical sensitivity and predicted diagnostic sensitivity. Our data indicated that this assay is a good alternative to currently used singular assays for SVV-1 or FMDV detections in at least three aspects: 1) increased diagnostic coverage to filed FMDV and SVV-1 strains; 2) one PCR reaction detects and differentiates SVV-1 from FMDV infections; and 3) the inclusion of the 18S rRNA internal control enables the monitoring of nucleic acid extraction efficiencies and potential PCR inhibitions, thus will help to reduce false negative results.

18. Validation of a Real-Time Reverse Transcription PCR Assay for Detection of Porcine Kobuvirus (PKV) in Porcine Diagnostic Samples (17-144)

Principal investigators	Phil Gauger (pcgauger@iastate.edu, tel. 515-294-1950) and Karen Harmon
Type of assay	PCR
Background information	Objective #1: Develop and validate an rRT-PCR assay to detect the US strain(s) of porcine Kobuvirus (PKV) in swine feces, fecal swabs, and oral fluids; Objective #2: Develop and validate a sequencing protocol for VP1 based on the US PKV sequences for primer targets; Objective #3: Validate the rRT-PCR developed at the ISU VDL using clinical samples from swine in China to confirm the ability to detect Chinese strains of PKV.
Sample type	Oral fluid, feces, fecal swabs
Analytical sensitivity	0.2 copies/µl, Ct=35
Analytical specificity	100%
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	No information; contact principal investigator
Other	10/37 fecal samples and 13/75 tissue homogenates were rRT-PCR + with ISU VDL PDV assay compared to 8/37 fecal samples and 8/75 tissues homogenates tested with the gel-based assay targeting Chinese strains of PKV.

Abstract: Porcine kobuvirus (PKV) is a small, non-enveloped, single-stranded positive sense RNA virus in the family Picornaviridae in the genus kobuvirus. Porcine kobuvirus RNA has been detected in healthy swine, although recent reports suggest PKV may cause diarrhea, dehydration, and vomiting in pigs in Asia. Due to the high prevalence of PKV in China and other Asian countries, swine producers in the United States (US) have increasing concerns regarding the presence and impact of PKV in the US. To facilitate diagnosis and monitoring for the presence of this virus, reliable detection methods and sequencing assays are needed. The objective of this study was to validate a real time reverse transcription PCR (rRT-PCR) to detect the presence of PKV in swine based on US strains of the virus. The rRT-PCR targets the 5' untranslated region (5' UTR) of the genome and was designed using 38 PKV sequences available in GenBank. Five whole genome sequences were generated from porcine diagnostic cases submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) to facilitate rRT-PCR and sequencing design. Approximately 1845 feces, fecal swabs, and oral fluids swine samples submitted to the ISUVDL were screened for PKV using the new rRT-PCR assay. Nucleic acid was extracted using the MagMAX Pathogen RNA/DNA kit from Life Technologies and amplification conducted on the Applied Biosystems 7500 Fast instrument using TaqMan Fast Virus 1-step Master Mix reagents. Sequencing the VP1 and 3D gene was attempted on PKV rRT-PCR positive samples with a Ct value less than 24 to assess the accuracy of the rRT-PCR assay. Approximately 100 sequences of each PKV target were generated and followed by confirmation using the BLAST feature in GenBank. Isolates of various enteric pathogens including porcine reproductive and respiratory syndrome virus (PRRSV) types 1 and 2, transmissible gastroenteritis virus, porcine

deltacoronavirus, *Lawsonia intracellularis*, non-INDEL and INDEL strains of porcine epidemic diarrhea virus (PEDV), *Salmonella enteritidis*, and *Escherichia coli* were analyzed using the assay to verify its specificity. In vitro transcribed RNA standards were generated to determine the limit of detection. Analytic sensitivity of the assay at Ct of 35 corresponded to detection of 0.2 copies/µl. Porcine samples were used to confirm detection of Chinese strains of PKV using the ISU VDL rRT-PCR assay. Validation of an rRT-PCR will assist the diagnosis, surveillance and monitoring of PKV in US swine and facilitate future studies to evaluate the pathogenesis of PKV.

19. Case Investigation and Development of Improved Diagnostics for Porcine Sapovirus (19-220)

Principal investigator	Ganwu Li (liganwu@iastate.edu, tel. 515-294-3358)
Type of assay	PCR
Background information	Objective 1: Further confirm the role of porcine sapovirus as the sole etiological agent of diarrhea in the case described. Objective 2: Determine the incidence of porcine sapovirus in swine herds of the United States.
Sample type	Feces, intestinal tissues
Analytical sensitivity	5 copies/reaction
Analytical specificity	100% (Ct>40)
Diagnostic sensitivity	No information; contact principal investigator
Diagnostic specificity	Porcine SaV genogroup III
Other	In 200 pigs from outbreak farm: Pigs with diarrhea, Ct (15.9 ± 0.59); clinically healthy pigs, Ct (35.8 ± 0.71) National study: Pigs with diarrhea - 45.3% positive, Ct=26.0± 0.5; Clinically healthy pigs - 43.1% positive, Ct=33.2 ± 0.9

Abstract: Porcine SaV was first identified by electron microscopy in the United States in 1980 and since then, it has been reported from both asymptomatic and diarrheic pigs usually with mixed infection of other enteric pathogens. SaV as the sole etiological agent of diarrhea in pigs has, until this time, never been reported. Here, we use of four independent lines of evidence metagenomics analysis, real-time RT-PCR, histopathology, and in situ hybridization and confirm that porcine sapovirus of genogroup III as the cause of the enteritis and diarrhea. A highly sensitive and specific real-time RT-PCR was established to detect porcine sapovirus of genogroup III. Examination of 200 fecal samples from the outbreak farm showed that those pigs with clinical diarrhea had significantly lower Ct values (15.9 ± 0.59) than those from clinically healthy pigs (35.8 ± 0.71) with 1024-fold changes. Further survey of 336 fecal samples from different states across over the United States demonstrated that the samples from pigs with clinical diarrhea had a comparable positive rate (45.3%) with those from non-clinical pigs (43.1%). However, the SaV-positive pigs with clinical diarrhea had significantly higher viral loads (Ct=26.0± 0.5) than those positive pigs of clinically healthy (Ct=33.2 ± 0.9). These results suggest that porcine sapovirus III play an important role in causing swine enteritis and diarrhea and rRT-PCR is a reliable method to evaluate the pathogenicity role of porcine SaV. Our findings provide significant insights for a better understanding of the epidemiology and pathogenicity of porcine SaV.

Part B: Enzyme Linked Immunosorbent Assays (ELISAs)

1. Hepatitis E Virus ELISA in Serum and Oral Fluids (17-197)*

Principal investigator	Tanya LeRoith (tleroith@vt.edu, tel. 540-231-7627)
Type of assay	ELISA
Background information	Objective 1. Validation of an immunoassay (PrioCHECK ELISA) to detect anti-swine HEV antibodies in oral-fluids; Objective 2. Evaluation of the diagnostic performance of the ELISA in commercial swine.
Sample type	Serum, oral fluids
Analytical sensitivity and specificity	HEV genotype 3: 10/10 pigs that were experimentally inoculated were positive via ELISA using serum; 0/10 pigs were positive via ELISA using oral fluids HEV genotype 4: 10/10 pigs that were experimentally inoculated were positive via ELISA using serum; 0/10 pigs were positive via ELISA using oral fluids
Diagnostic sensitivity and specificity	In commercial swine, 175/304 pigs were positive for HEV via ELISA using serum; 129/304 pigs were negative for HEV via ELISA using serum Anti-HEV Ab were detected in the oral fluid of one pig via ELISA

*Test not currently available

Abstract: The research outlined in this proposal was designed to validate the use of an oral fluid-based diagnostic test for rapid swine HEV detection in the United States. To assess the use of the diagnostic test, in objective 1, we proposed to validate the PrioCHECK HEV Ab Porcine ELISA Kit from Life Technologies Limited, United Kingdom (Cat #4600010) indirect ELISA and examine if the test has the capacity to distinguish a range of antibody concentrations with a minimum background activity in oral fluids. In this objective, we experimentally infected pigs with genotype 3 and genotype 4 swine HEV to attempt to validate the commercially available assay for use in a new matrix. Full-length ORF3 polypeptides from swine (genotype 4) and human (genotype 1) HEV have 76% identity but since HEV has only one serotype, we hypothesized that the commercial ELISA would be able to detect both genotypes. Serum was used as a method comparison. Paired serum dilutions from infected pigs were used to determine the efficiency and limit of detection. Pigs inoculated with PBS served as negative controls. In order to confirm viral infection, fecal virus shedding was monitored by swine HEV-specific nested RT-PCR. We use nested PCR because it more reliably detects low levels of fecal shedding in our hands. All pigs were negative for swine HEV RNA by RT-PCR or swine HEV antibodies by ELISA prior to inoculation in both animal studies. PrioCHECK ELISA was able to detect antibodies in the serum of pigs infected with both genotypes. However, no anti-HEV antibodies were detected in the oral fluids from these pigs. Seroconversion of HEV antibody was first detected at 3 wpi in group 2 pigs and at 4 wpi in group 1. The pigs in control groups remained seronegative throughout the study.

In objective 2, we proposed to evaluate the use of the assay in detecting HEV infection in oral fluids in commercial pigs. In order to show that the results in the first study are reliable and replicable, we evaluated the ability of the ELISA to detect HEV antibodies in oral fluids of commercial pigs. Paired blood and oral fluid samples were collected from 304 pigs from a commercial source. The ELISA results showed that 175 out 304 pigs had anti-swine HEV antibodies in serum. The remaining 129 pigs had no serum anti-swine HEV antibody. Anti-swine HEV antibodies were detected in oral fluids of only one pig. An IgA ELISA was used in a separate test to determine if anti-HEV antibodies in oral fluids were IgA rather than IgG. No anti-HEV antibodies were detected in any of the pigs regardless of the serum status.

2. Development and Validation of ELISA to Detect IgA, IgM, IgG in Serum and Oral Fluids to Porcine Sapelovirus (PSV) (17-198)*

Principal investigator	Doug Marthaler
Current contact	TBD
Type of assay	ELISA
Background information	Objective: to develop a PSV indirect ELISA assay (based on PSV VP2-VP1) for serosurveillance in serum and oral fluids, and to generate monoclonal antibodies (mAb) against VP1 and VP2 proteins for viral detection.
Sample type	Serum, oral fluids
Analytical sensitivity and specificity	VP2-VP1 (combined) had the highest OD reading, and a higher antibody detection with 300 ng of protein (compared to VP1 only and VP2 only)
Diagnostic sensitivity	99.2% (cutoff 0.6075)
Diagnostic specificity	97% (cutoff 0.6075)
Other	PSV antibody response was investigated in post-weaning pigs naturally infected with PSV, which increased until 28 days post exposure.

*Test not currently available

Abstract: Porcine sapelovirus (PSV) can cause both symptomatic and asymptomatic diseases. Recently, PSV has been reported to be the etiologic agent of polioencephalomyelitis in pigs from the United States. However, indirect ELISAs are lacking for epidemiological surveillance and disease control. In this study, a PSV VP2-VP1-based indirect ELISA assay was developed for serosurveillance. To determine the optimal cutoff for indirect PSV ELISA, a total of 604 serum samples (503 positives and 101 negatives) from previous experimental studies were employed. The optimal cutoff of 0.6075 maximized the diagnostic sensitivity and diagnostic specificity of 99.2% and 97.0%, respectively. Using a single lot of internal control serum, PSV ELISA exhibited a within-plate Coefficient of Variance (CV) of 5.13%, within-run CV of 5.96%, and between-runs CV of 7.73%, indicating the PSV ELISA was highly repeatable with serum samples. In additional, a panel of monoclonal antibodies (mAb) against VP1 and VP2 proteins were generated for viral detection. Finally, the PSV antibody response was investigated in post-weaning pigs naturally infected with PSV, which increased until 28 days post exposure. The indirect PSV ELISA and specific mAbs provide powerful tools for rapid diagnostic testing for disease control and prevention.

3. Development and Validation of ELISA to Detect IgA, IgM, and IgG in Serum and Oral Fluids to Porcine Teschovirus (PTV) (17-199)*

Principal investigator	Doug Marthaler
Current contact	TBD
Type of assay	ELISA
Background information	Objective: to develop and validate a pan-PTV indirect ELISA for diagnostics.
Sample type	Serum, oral fluids
Analytical sensitivity and specificity	VP2-VP1 linked protein had the highest OD reading (compared to VP1 only and VP2 only)
Diagnostic sensitivity	99.2% (cutoff 0.4105)
Diagnostic specificity	98.4% (cutoff 0.4105)
Other	PTV antibody response in post-weaning piglets was positive at day 35 and peaked on day 63. Using oral fluids, indirect ELISA did not detect PTV Ab. Using FMIA to increase sensitivity in oral fluids, no Ab was detected during first 7 days; sporadic detection occurred from days 35–41; no PTV Ab was detected after 41 days.

*Test not currently available

Abstract: Porcine teschovirus (PTV) was first identified in pigs exhibiting various symptoms including ataxia, nystagmus, convulsions, polioencephalomyelitis, and paralysis. The lack of pan-PTV ELISA (ability to detect all the serotypes) hampers our ability to conduct PTV surveillance and determination of the immune status within a herd. We created three protein constructs (VP1, VP2, and VP2-VP1 linker), and the VP2-VP1 linker protein generated the highest fluorescence levels for antibody detection. The diagnostic sensitivity and specificity were determined using the known PTV antibody status of 558 serum samples (369 positive and 189 negatives), and assay had a diagnostic sensitivity and specificity of 99.2% and 98.4%, respectively. Using a single lot of internal control serum, the PTV ELISA exhibited a within-plate Coefficient of Variation (CV) of 7.32%, within-run CV of 7.85%, and between-runs CV of 9.24%. The indirect PTV ELISA was employed to investigate the immune response in post-weaned piglets after PTV exposure, and the ELISA detected PTV antibodies on day 35. The detection of antibodies peaked on the last day of the study (63 days). Fluorescent microsphere immunoassay (FMIA) on the BioRad system was used to detect PTV antibodies in oral fluids. However, the ability to detect PTV antibodies in oral fluids was low and highly variable, suggesting oral fluids may not be a suitable specimen to determine the immune status of a herd. In conclusion, we developed and validate an indirect PTV ELISA for use in a diagnostic setting and measured the antibody response to PTV in weaned piglets.

4. Development of Antibody Detection Assays for Swine Influenza B, C, and D Viruses (17-203)

Principal investigator	Xuming Liu
Current contact	Jianfa Bai (jbai@vet.ksu.edu, tel. 785-532-4332)
Type of assay	ELISA
Background information	Objective 1: Identification and expression of antigens that are specific and conserved within each influenza virus of IBV, ICV, and IDV. 1) Identification of candidate antigens of IBV, ICV, and IDV; 2) Production of IBV, ICV, and IDV antigens by cloning of target viral genes and expression of recombinant proteins. Objective 2: Development of serum- and oral fluid-based ELISAs for detection of antibody responses against IBV, ICV and IDV infection in pigs. 1) Standard serum and oral fluid production using pigs experimentally infected with IBV, ICV, and IDV; 2) ELISA development, test condition optimization, and initial analytical validation. Objectives 3: Diagnostic validation of serum- and oral fluid-based ELISAs with experimental and field swine samples. 1) Determination of cutoff values, diagnostic sensitivity and specificity; 2) Measurement of repeatability.
Sample type	Serum, oral fluids
Analytical sensitivity and specificity	LODs for the high-level positive clinical serum samples or standard controls were 1:3200 (IBV), 1:800 (ICV), and 1:1600 (IDV) Specificity 100% (serum, for IBV, ICV, and IDV)
Diagnostic sensitivity and specificity	IBV, sera-IgG: sensitivity = 97.83%, specificity = 98.36% (cutoff 0.07) IBV, oral fluids-IgG: sensitivity = 90.48%, specificity = 100% (cutoff 0.22) ICV, sera-IgG: sensitivity = 88.24%, specificity = 95.83% (cutoff 0.18) IDV, sera-IgG: sensitivity = 95.65%, specificity = 98.11% (cutoff 0.08) IBV, sera-IgA: sensitivity = 94.74%, specificity = 100% (cutoff 0.04) IBV, oral fluids-IgA: sensitivity = 94.12%, specificity = 95.45% (cutoff 0.08) IBV, sera-IgM: sensitivity = 96%, specificity = 96.15% (cutoff 0.07)

	Note for below: samples with S/P ratio >1.5 times cutoff value considered positive; samples with S/P ratio 1-1.5 times cutoff value considered weak positive/suspect positive IBV: 173/441 (39.2%) of clinical serum samples were positive for IBV; 74/441 (16.8%) of clinical serum samples were weak/suspect positive 19/185 (10.3%) of clinical oral fluid samples were positive for IBV; 27/185 (14.6%) of clinical oral fluid samples were weak/suspect positive ICV: 194/441 (44%) of clinical serum samples were positive for ICV; 79/441 (17.9%) of clinical serum samples were weak/suspect positive IDV: 176/441 (39.9%) of clinical serum samples were positive for IDV; 81/441 (18.4%) of clinical serum samples were weak/suspect positive

Abstract: Influenza is a highly contagious viral respiratory disease caused by influenza A, B, C, and D viruses (IAV, IBV, ICV, and IDV). The objective of this study was to develop enzyme-linked immunosorbent assays (ELISAs) for the detection and monitor of swine antibody responses against IBV, ICV, and IDV infections. Serum samples and oral fluids collected from experimentally inoculated piglets and from clinically confirmed field swine samples were used to generate IBV, ICV, and IDV positive and negative antibody standards. The highly immunogenic influenza virus nucleoprotein (NP) was selected as potential antigen for ELISA development for the antibody detection. Both the synthesized potentially immunogen peptides of NP and the full-length NP proteins expressed in *E. coli* were used for ELISA tests. Five or six peptides in each of influenza virus NP proteins that were predicted to be potentially immunogenic were synthesized and tested with experimental samples, but no good signals were generated from the ELISA tests. Indirect ELISAs with expressed full-length NP proteins of IBV, ICV, and IDV have been successfully developed. Optimized full-length NP genes of IBV (coding 560aa NP), ICV (565aa), and IDV (552aa) were synthesized and cloned into expression vector pET-28a (+) and transformed into the *E. coli* protein expression strain BL21(DE3). The expressed His-tagged recombinant NP protein sizes of IBV, ICV and IDV are approximate 66.69 kDa, 68.85 kDa and 66.32 kDa, respectively. Indirect ELISA procedure and conditions were optimized by using purified NP proteins, standard positive/negative antibody samples, and goat anti-swine IgG labeled with horseradish peroxidase (HRP) as secondary antibody. The OD_{405} detected from positive IBV, ICV, and IDV serum samples were up to 3.2, 1.4 and 2.3, respectively. The cutoff values were determined by ELISA tests on swine serum and oral fluid samples according to the sample-to-positive (S/P) ratios of OD_{405}. The tests were validated with 441 serum samples and 185 oral fluid samples. Our data showed that IgG is the main type of antibody in response to IBV, ICV, and IDV in both serum and oral fluid samples. Results indicated that the ELISAs can detect and differentiate animal antibodies in serum samples and oral fluids in response to the respective influenza virus, and have no cross-reactions among the antibodies in response to infections of influenza A, B, C, and D viruses. In conclusion, the ELISA developed in this study using recombinant NP proteins as antigen can sensitively detect and specifically differentiate swine antibodies in response to IBV, ICV, and IDV. This ELISA offers a useful diagnostic tool for antibody screening and follow-up diagnostics that is an integral part of epidemiological surveys and outbreak investigations, which can provide professional guidance to disease control and prevention.

5. Development and Evaluation of Antibody Detection Assay for PCV3 Virus (17-204)

Principal investigator	Jianfa Bai (jbai@vet.ksu.edu, tel. 785-532-4332)
Type of assay	ELISA
Background information	Objective 1: ELISA antibody test development for PCV3 1) Antigen production by cloning and expression of PCV3 capsid protein; 2) Standard serum and oral fluid production using pigs experimentally infected with PCV3; 3) Test condition optimization for PCV3 ELISA. Objective 2: Diagnostic validation of the PCV3 ELISA test 1) Positive and negative cutoff value determination; 2) Sensitivity, specificity and repeatability evaluation using serum and oral fluid samples with known infection status; 3) Comparison of sensitivity and specificity of PCV3 ELISA with that of PCV2 commercial ELISA kit.
Sample type	Serum, oral fluids
Analytical sensitivity and specificity	LODs=0.79ng pAb against PCV3-cap-2 per well, 1.65ng against PCV3-rep per well Specificity 100% (for both PCV3 and PCV2 peptide detection)
Diagnostic sensitivity and specificity	169/367 (46%) of clinical serum samples were positive (S/P cutoff 0.19) 90/276 (32.6%) of clinical oral fluid samples were positive (S/P cutoff 0.42)

Abstract: The objective of this research was to develop an ELISA antibody test to monitor animal responses to PCV3 infections. To differentiate antibody response to PCV2 infections, a PCV2 ELISA antibody test was developed as well to verify that the PCV3 test is specific to PCV3 and does not detect antibody response to PCV2 infections. Experimentally inoculated animals were used to generate PCV3 positive and negative standards for the ELISA assay development. Expressed PCV3 capsid protein was used first for the test development, but it did not generate good signal. Five peptides in the capsid and one from the rep gene region that were predicted to be potentially immunogenic were synthesized and tested with experimental samples. One of five capsid-based peptides and the peptide based on the rep gene were generating good signals. The two peptides were pooled for the PCV3 ELISA development. A PCV2 peptide in the capsid that were previously published (Hung and Cheng, 2017) and indicated to generate good signal was synthesized and used for PCV2 antibody detection and PCV3 antibody differentiation. The test was validated with 367 serum samples and 276 oral fluid samples. Our data indicated that IgG is the main type of antibody in both serum oral fluid samples, however, IgA has shown detectable signals in oral fluid samples, and IgM was detected in early stage of infections in our animal study. As addition of more antibodies did not increase the ELISA assay background level, pooled IgG, IgM and IgA was used for both serum and oral fluid diagnostic samples. The results indicated that the assay can specifically detect animal responses to PCV3 and PCV2 infections. The signal intensity generated on oral fluid was lower than that with serum samples, using signal to positive ratio we were able to detect antibody responses from oral fluid samples.

6. Development and Evaluation of a Dual Matrix Serum/Oral Fluid Atypical Porcine Pestivirus ELISA Using Known Status Samples (18-136)

Principal investigator	Bailey Arruda
Current contact	Luis Gimenez-Lirola (luisggl@iastate.edu, tel. 515-294-7025)
Type of assay	ELISA
Background information	Objective 1: Generation of a panel of specimens (serum and oral fluid) and specific polyclonal antibodies against atypical porcine pestivirus (APPV). Objective 2: Development and evaluation of oral fluid and serum antibody assay specific against APPV.
Sample type	Serum, oral fluids, nasal swabs
Diagnostic sensitivity and specificity	Specificity 100% for both serum (cutoff ≥0.10) and oral fluid (cutoff 0.15) E^{rns} iELISA assays
Other	RT-qPCR testing for Objective 1: APPV was first detected at 10 DPI in the serum of four inoculated animals. By 14 and 17 DPI, APPV was detected in the serum of 69% and 81% of APPV-inoculated pigs, respectively. By 35 DPI, APPV was detected in the serum of every APPV-inoculated animal and all animals remained positive until the end of the study at 70 DPI. APPV was detected in 63% and 83% of oral fluids at 14 and 17 DPI, respectively. By 24 DPI, all oral fluid samples were positive and remained positive until the end of the study at low Cq values. APPV was first detected in nasal swabs from 4 animals at 17 DPI with nasal shedding detected in animals at 42, 49, and 56 DPI.

Background: Atypical porcine pestivirus (APPV) is the most common cause of congenital tremor (CT) in pigs. APPV is transmitted from the dam to fetuses resulting in all or a subset of piglets with CT. Gilt and low parity sows are frequently reported to have CT litters; however, CT litters can be seen with multiparous sows as well. Regardless of parity, CT litters are likely due to insufficient dam immunity at a critical timeframe of gestation. There is limited information concerning the epidemiology and pathophysiology of APPV. Currently, there is no available serologic assay. Such an assay would provide meaningful information to assess the effectiveness of preventative measures such as acclimation and vaccination as well as improve our understating of the infection dynamics and herd impact of APPV.

Methods: Twenty two cesarean derived colostrum deprived (CDCD), crossbred, mixed-sex, 6-week-old pigs were individually identified, blocked by litter and randomly assigned to one of two groups in separate rooms based on inoculum and pen. Two pigs were placed per pen with three pens (negative control animals) or eight pens (positive control animals) per room. Oral fluids, serum, and nasal swab samples were collected prior to challenge and submitted for detection APPV by PCR prior to inoculation. Pigs were inoculated with MEM (n=6; negative control) or APPV (n=16) intravenously, intramuscularly and intranasally. Serum, pen-based oral fluids samples (3 or 8 pens/group), and individual nasal swabs were collected 0, 3, 7, 10, 14, 17, 21, 28, 35, 42, 49, 56, 63, and 70 days post inoculation (DPI). Oral fluids were also collected 31, 38, 45, 52, 59 and 66 DPI. A selected immunogenic region of APPV Erns was cloned, expressed, and purified as a recombinant polypeptide. The purified protein were dialyzed against phosphate-buffered saline (PBS) (20 mM phosphate buffer and 150 mM NaCl, pH 7.4) and analyzed by SDS-PAGE and Western blot. Horseradish peroxidase (HRP)-conjugated goat anti-pig IgG secondary antibodies were used for the indirect ELISA format.

Results: APPV was first detected at 10 DPI in the serum of four inoculated animals (mean Cq value [SD]: 32.5[1.3]). By 14 and 17 DPI, APPV was detected in the serum of 69% (28.4[4.2]) and 81% (26.1[3.8]) of APPV-inoculated pigs, respectively. By 35 DPI, APPV was detected in the serum of every APPV-inoculated animal and all animals remained positive until the end of the study at 70 DPI. APPV was detected in 63% (31.9[3.3]) and 83% (25.3[5.9]) of oral fluids at 14 and 17 DPI, respectively. By 24 DPI, all oral fluid samples were positive and remained positive until the end of the study at low Cq values (17.3-20.0). APPV was first detected in nasal swabs from 4 animals at 17 DPI (31.2[0.6]) with nasal shedding detected in animals at 42, 49, and 56 DPI. APPV was not detected in any sample type from negative control pigs. The serum and oral fluid Erns iELISA assays have a 100% diagnostic specificity at a cut-off ≥0.10 and 0.15, respectively.

Discussion: This is the first study to experimentally infect swine with APPV and monitor the infection dynamics overtime out to 70 DPI. The results of this experimental inoculation provide evidence that APPV viremia can be prolonged, at least 60 days could be expected following intentional exposure. These finding may be applicable if gilt acclimation protocols are undertaken. Additionally, based on the results of this study it appears that oral fluid is an appropriate and likely highly sensitive sample type to monitor herd status by RT-qPCR. Lastly, both oral fluid and serum iELISA assays can be used to evaluate individual and herd status prior to and following intervention strategies. This project provided important foundational knowledge concerning the infection dynamics of APPV in experimentally infected swine while also providing the necessary samples to develop and evaluate serologic assays that will assist in furthering our understanding APPV and preventing CT litters.