RESEARCH REPORT
SWINE HEALTH

Title: **Ex-vivo bioassay method to assess viral infectivity in feeds and non-traditional sample matrices - NPB #14-160**

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Industry Summary

Porcine epidemic diarrhea (PED), caused by a coronavirus (PEDV), is a newly emerged enteric disease of swine, which was confirmed in the United States for the first time in April 2013. Since then, PED has caused unprecedented challenges to the US swine industry due to an extremely high mortality (up to 100%) in pre-weaning pigs and significant productivity loss in growing and breeding pigs. While still unanswered how PEDV was initially introduced to US swine, imported feed ingredient(s) has been suspected as a source for introduction to the US. Feedstuff has also been identified as a contributing factor to spreading of PEDV within the US. While positive PCR finding of PEDV RNA in feed or feedstuffs raised the serious question about the cleanness of environment at both collection and manufacturing sites and manufacturing quality control processes of raw materials to supply and storage of final products, infectivity of such PCR-positive materials in pigs has been extremely difficult to prove through feeding trials. Knowing that PCR-based tests detects the presence of targeted genetic material regardless of live or dead, a different and reliable way to assess the infectivity of these materials was necessary.

PEDV has been known to be difficult to isolate or propagate in cell culture (a 5-6% success rate). Furthermore, complete feed, feed ingredients or environmental samples are not matrices which work well in a cell culture system due to cytotoxicity. All these facts reduce the value of cell culture-based testing to assess if a material in question contains an infectious virus. Alternatively, swine bioassay is an excellent tool to access infectivity as pigs are the natural hosts. However, swine bioassay is resource-driven (i.e., expensive) and a biologically variable system which can be confounded by many factors, not to mention its longer turnaround. The following proposed study was for a proof-of-concept to address the need of diagnostic procedures for determining potential live virus contamination of feed or feedstuffs. Since small intestine is the ultimate place for enteric viral pathogens to replicate, leading to the disease due to functional disruption of villi and other parts, we explored an ex-vivo bioassay for PEDV in feed or feedstuffs using "*Tied Small Intestinal Segment* (TSIS)" by maintaining the intact structure of intestine outside of the pig under laboratory conditions. As neonates are born with intestinal tissues with fully functional enterocytes which are the most susceptible cells to PEDV, the specific objective of the study is to determine if TSIS from neonatal pigs will be a cost-effective diagnostic tool for rapid and reliable determination of infectivity of PEDV.

A tied small intestinal segment (TSIS) was a cross-sectional piece of small intestine (6-10 cm in length) tied-off and liquid material (i.e., inoculum) injected into the lumen. The TSIS was kept moist by immersing

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in sterile cell culture media within a sterile petri dish and maintained at 37°C in a humidified CO_2 incubator to mimic the body condition. Hence TSIS is a miniaturized natural pig gut with functional enterocytes along with villous structure. Intestinal segments from CDCD or snatch-farrowed piglets obtained from PEDV and TGEV negative farms were used of the TSIS. To supply sufficient number of TSIS for the study, piglets of the same litter were used as intestine donor. Three separate experiments were conducted to address the following qusetions:

1. How long TSIS can be maintained *in vitro* without detrimental loss or alteration of villous structure?
2. Is TSIS permissive to coronaviruses and rotaviruses and all intestinal segments are equally susceptible to these viruses?
3. How sensitive is TSIS method for viral infectivity assay?

Tied small intestinal segments could be maintained *in vitro* up to 72 hours without significant loss of enterocytes and villous structure under optimized conditions regardless of sample matrices (i.e., media or feed extracts) tested. Virus infection and growth in TSIS was evident to a degree by PCR or IHC staining when inoculated with 2ml cell-culture derived PEDV or TGEV containing as low as 10^2 PFU/ml at 72 hours post inoculation (PI) but not at 48 hours PI. Such an evidence of virus growth was not observed in TSIS inoculated with swine influenza virus at a rate of up to 10^6 $TCID_{50}$/ml within 72 hours. Jejunum and ileum were permissive to both coronaviruses regardless of source of intestine (i.e., CDCD piglets or snatch-farrowed piglets). When feed or feed ingredients, which were positive for PEDV nucleic acid by PCR at Iowa State University Veterinary Diagnostic Laboratory, were tested in TSIS, no infectivity was detected. Some of the samples which were tested by swine bioassays also did not show the presence of infectious PEDV.

The study outcome suggests that TSIS can be an ex-vivo bioassay tool to measure the presence of an infectious enteric viral pathogen in feed or non-traditional sample matrices. While this new method would be better than cell-culture based assessment, further optimization and refining is necessary to significantly enhanced the sensitivity of TSIS-based testing for infectivity. Some examples are: a) the ability to maintain TSIS integrity for longer than 72 hours and b) availability of assays for tissues to demonstrate virus infection/replication in enterocytes which are more sensitive than IHC.

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Scientific Abstract

Virus-contaminated feed ingredients has been suggested as a source of porcine epidemic diarrhea virus (PEDV) to US and Canadian swine and as a contributing factor to rapid regional and national spreading of the virus. While positive PCR finding of PEDV RNA in feed or feedstuffs raised the serious question about the cleanness of environment at both collection and manufacturing sites and manufacturing quality control processes of raw materials to supply and storage of final products, infectivity of such PCR-positive materials in pigs has been proven extremely difficult through feeding trials, which raised the need for a different and reliable way to assess the infectivity of these materials. PEDV has been known to be difficult to isolate or propagate in cell culture (less than 10% success rate). Alternatively, swine bioassay is an excellent tool to access infectivity as pigs are the natural hosts. However, swine bioassay is resource-driven (i.e., expensive and labor-intensive), is a biologically variable system which can be confounded by many factors, and takes longer turnaround.

Small intestine is the ultimate place for enteric viral pathogens to replicate, leading to the disease due to functional disruption of villi and other parts. We explored an ex-vivo bioassay for PEDV in feed or feedstuffs using "*Tied Small Intestinal Segment* (TSIS)" by maintaining the intact structure of intestine outside of the pig under laboratory conditions. A TSIS was a cross-sectional piece of small intestine with both ends tied-off and liquid material (i.e., inoculum) injected into the lumen, which was then kept moist by immersing in sterile cell culture media within a sterile petri dish and maintained at 37°C in a humidified CO_2 incubator to mimic the body condition. Hence TSIS could be considered to be a miniaturized natural pig gut with functional enterocytes along with villous structure. The specific objective of the study was to determine if TSIS from neonatal pigs can be a cost-effective diagnostic tool for rapid and reliable determination of infectivity. Specific aims were:

1. To assess how long TSIS can be maintained in-vitro without detrimental loss or alteration of villous structure;
2. To determine if TSIS permissive to coronaviruses and rotaviruses and all intestinal segments are equally susceptible to these viruses; and
3. To characterize how sensitive is TSIS method for viral infectivity assay.

Each TSIS could be inoculated with pp to 2ml of sample. TSIS could be maintained *in vitro* up to 72 hours without significant loss of enterocytes and villous structure under optimized conditions regardless of sample matrices (i.e., media or feed extracts) tested. Virus infection and growth in TSIS was evident to a degree by PCR or IHC when inoculated with cell-culture derived PEDV, TGEV or porcine rotavirus A containing as low as 10^2 PFU/ml at 72 hours post inoculation (PI) but not at 48 hours PI. Jejunum and ileum were permissive to the viruses tested regardless of source of intestine (i.e., CDCD or snatch-farrowed piglets). When feed or feed ingredients positive for PEDV RNA with Ct values of 28-34 were tested in TSIS, no infectivity was detected. Some of the samples which were tested by swine bioassays also did not show the presence of infectious PEDV.

In conclusion, this proof-of-concept study suggests that TSIS could be an ex-vivo bioassay tool to measure the presence of infectious viruses in feed or feedstuffs. While this new method appeared to be better than cell-culture based assessment, TSIS method was not sensitive enough under study conditions to detect the presence of PEDV at a low level which has been commonly the case with any of PEDV RNA positive feedstuffs tested at ISUVDL based on Ct values. Therefore, further optimization remains to enhance the sensitivity of TSIS-based testing for infectivity.

Introduction

Porcine epidemic diarrhea (PED), caused by a coronavirus (PED virus), is a newly emerged enteric disease of swine, which was confirmed in the United States for the first time in April 2013. Since then, PED has caused unprecedented challenges to the U.S. swine industry due to an extremely high mortality (up to100%) in pre-weaning pigs and significant productivity loss in growing and breeding pigs. To date, the disease has been identified in 38 states with estimated losses of greater than 4 million pigs since first year.

While still unanswered how PED virus (PEDV) was initially introduced to US swine, feed ingredients imported from PED-endemic country have been suspected as a source for introduction of the virus to the US. Once PEDV emerged in the US, the rate of virus spread amongst swine operations has been much faster than expected, even with tremendous effort to contain through enhanced biosecurity or intentional controlled exposure of animals to the virus (a.k.a., feedback) for herd immunity against PEDV. The fact that US swine were naïve to PEDV is a reason for rapid spread of the virus. Transportation of contaminated pigs and suboptimal truck washing, as well as virus-contaminated feedstuffs, has also been identified as a contributing factor to virus spreading and/or disease outbreaks.

While positive PCR finding of PEDV RNA in feed or feedstuffs raised the serious question about the cleanness of environment (both collection and manufacturing sites) and manufacturing quality control processes of raw materials to supply and storage of final products, infectivity of such PCR-positive materials in pigs has been proven extremely difficult through feeding trials. Knowing that PCR-based tests detects the presence of targeted genetic material regardless of live or dead, a different and reliable way to assess the infectivity of these materials is necessary.

PEDV has been known to be difficult to isolate or propagate in cell culture, currently about a 5-6% success rate on clinical samples at the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL). Such difficulty is also applicable to other enteric viral pathogens such as rotaviruses, other coronaviruses, caliciviruses, and so on. Furthermore, complete feed, feed ingredients or environmental samples are not matrices which work well in a cell culture system due to cytotoxicity. All these facts reduce the value of cell culture-based testing to assess if a material in question contains an infectious virus. Alternatively, swine bioassay is an excellent tool to access infectivity as pigs are the natural hosts. Pig infection is indeed the ultimate answer for questionable materials. Nonetheless, swine bioassay is resource-driven (i.e., expensive and labor-intensive) and a biologically variable system which can be confounded by many factors, not to mention its longer turnaround. Hence, the following proposed study was conducted as a proof-of-concept to address the need of diagnostic procedures for determining potential live virus contamination of non-traditional sample matrices such as feed or feedstuffs.

Objectives

Small intestine is the ultimate place for enteric viral pathogens to replicate, leading to the disease due to functional disruption of villi and other parts. Neonates are born with intestinal tissues with fully functional enterocytes which are the most susceptible cells to all known enteric viral pathogens including PEDV. We explored an ex-vivo bioassay for PEDV in feed or feedstuffs using "*Tied Small Intestinal Segment* (TSIS)" by maintaining the intact structure of intestine outside of the pig under laboratory conditions. The specific objective of the study was to determine if TSIS from neonatal pigs can be a cost-effective diagnostic tool for rapid and reliable determination of infectivity. Specific aims were:

4. To assess how long TSIS can be maintained in-vitro without detrimental loss or alteration of villous structure;
5. To determine if TSIS permissive to coronaviruses and rotaviruses and all intestinal segments are equally susceptible to these viruses; and

6. To characterize how sensitive is TSIS method for viral infectivity assay.

Materials & Methods

A "tied small intestinal segment" (TSIS) was a cross-sectional piece of small intestine (approximately 5-10 cm in length) with both ends tied-off and liquid material containing potentially infectious virus (i.e., inoculum) injected into the lumen. The TSIS was kept moist by immersing in sterile cell culture media within a sterile petri dish and maintained at 37°C in a humidified CO_2 incubator to mimic the body condition. Hence TSIS was considered to be a miniaturized natural pig gut with functional enterocytes along with villous structure and was used to address the specific aims. Intestinal segments from cesarean-derived/colostrum (CDCD)-deprived or snatch-farrowed piglets were used of the TSIS. Donor pigs were obtained from PEDV- and TGEV-negative farms. To supply sufficient number of TSIS for the study, piglets of the same litter were used as intestine donor. Each pig was used as a block for treatment (i.e., concentration, viruses, or viral titers) for statistical analysis. Each experiment below was repeated at least once whenever necessary to assess the reproducibility and/or the best estimate of sensitivity.

Experiment 1: *How long TSIS can be maintained in-vitro without detrimental loss or alteration of villous structure?*

Several TSIS from duodenum, jejunum and ileum of the same pig were made and inoculated with 1-2 ml of either cell culture media or feed extract. To prepared feed extracts, pelleted or milled feed was suspended in a cell culture medium at the concentration of 10, 20 and 50% (w/v) and vortexed on an orbital shaker for 20 min. After centrifuging at 500 rpm for 10 min, supernatants were used as feed extracts. Each inoculated TSIS was retained in cell culture environment for up to 96 hours and then fixed in formalin for histological evaluation at the end of each incubation time.

Experiment 2: *Is TSIS permissive to coronaviruses and rotaviruses and all intestinal segments are equally susceptible to these viruses?*

TSIS made from small intestines of the same pig was inoculated with cell-culture derived PEDV, TGEV and/or porcine rotavirus A (PoRV-A) at 10^3 PFU/ml (also with known PCR Ct value) and maintained for the longest survival time determined by Experiment 1. The viral dose was selected for the initial assessment based on previous neonatal pig study for PEDV and rotaviruses demonstrating that pigs developed the disease when given such a dose. At every 24 hours, fluid was harvested from TSIS for virus titration and PCR, and tissues were fixed in formalin for immunohistochemistry (IHC) staining to determine virus infection and growth. In addition to virus-free cell culture media (i.e., sham control), SIV isolate was used as virus negative control since these viruses did not expect to be replicated in intestinal tissues.

Experiment 3: *How sensitive is TSIS method for viral infectivity assay?*

A dose-response study was performed to answer this question. For this study, only PEDV was used. A set of 10-fold serial dilutions of PEDV isolate from 10^3 to 10^{-3} PFU/ml were prepared and tested by PCR to determine Ct values. One ml of each virus preparation was inoculated into each TSIS made from different sections of the small intestine. Inoculated TSIS was maintained in cell culture environment as described above. At every 24 hours, fluid from TSIS was harvested for virus titration and PCR and tissues be fixed in formalin for IHC staining for PEDV.

Results

Experiment 1: Each TSIS could be inoculated with pp to 2ml of sample. TSIS could be maintained *in vitro*

up to 72 hours without significant loss of enterocytes and villous structure under optimized conditions regardless of sample matrices (i.e., media or feed extracts) tested regardless of source of intestine (i.e., CDCD piglets or snatch-farrowed piglets). Longer incubation caused either bacterial growth or detachment of villi along with death of significant number of enterocytes on villous tips.

Experiment 2: Infection and growth of PEDV, TGEV and PoRV-A in TSIS was evident to a degree by both PCR and IHC staining, which were inoculated with 2ml cell-cultured media containing 10^3 PFU_{50}/ml of each virus at 72 hours but not at 48 hours post inoculation. No evidence of SIV infection and growth was observed within 72-hour incubation period.

Among intestinal segments, jejunum and ileum were most permissive to both coronaviruses and PoRV-A regardless of source of intestine (i.e., CDCD piglets or snatch-farrowed piglets). No evidence of virus infection and growth was detected in cecum/colon segment. It was inconclusive if duodenum segment supported virus infection and growth under study conditions.

Experiment 3: While PCR testing of harvested fluids suggested that infection and growth of PEDV in TSIS when inoculated with 10^1 PFU/ml, virus growth in enterocytes was only visible by IHC when TSIS was inoculated with 10^2 PFU/ml or higher of PEDV. The number of positive enterocytes were very scant though.

As an additional experiment, feed or feed ingredient samples (n=25) which were submitted to ISUVDL and tested positive for PEDV by PCR were tested in TSIS. Ct value of the samples ranged from 28 to 34. No infectivity was detected from any of the samples tested in TSIS system. Some of the samples were tested by swine bioassay, which was negative too.

Discussion

Outcomes of this proof-of-concept study suggest that TSIS could be an ex-vivo bioassay tool to measure the presence of infectious enteric viral pathogens in feed or non-traditional sample matrices as infection/growth of PEDV or TGEV in TSIS was detectable by laboratory assay such as PCR and IHC. While this new method appeared to be much better than cell-culture based assessment, TSIS method was not sensitive enough to detect the presence of PEDV at a low level which has been the case with any of PEDV RNA positive feedstuffs tested at ISUVDL based on Ct values under conditions presented in the current study.

The observed suboptimal sensitivity of TSIS method in this study may be attributed to the fact that TSIS could not be maintained longer than 72 hours without significant loss of enterocytes and villous structure. For some reason, all enteric pathogens used in the study did not replicate in this ex-vivo system rapidly as seen in neonatal pigs experimentally infected with those viruses. As a consequence, detection of virus infection/growth in TSIS particularly by IHC was severely hampered as IHC is less sensitive than PCR test and requires virus replication to a high level for positive detection. Therefore, further optimization and refining in these area may significantly enhance the sensitivity of TSIS-based testing for infectivity.

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