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Demonstration of Porcine Circovirus Type 2 Inactivation by the Low pH Step of the Trypsin Manufacturing Process Using a New Infectivity Assay

By Tara Tagmyer and Kathryn Martin Remington

Introduction

Porcine circoviruses (PCVs) are small (17 nm) non-enveloped viruses with a covalently closed, circular, single-stranded DNA genome.[1] PCV type 1 (PCV-1) and PCV type 2 (PCV-2) belong to the circovirus genus within the *Circoviridae* family. PCV-1 was originally isolated as a contaminant of porcine kidney (PK15) cells[2], and although it was found to be widely distributed in domestic swine in both North America and Europe, no correlation to any porcine disease or disorder has been established. PCV-2, however, has been found to be associated with several disease syndromes in pigs.[3]

For manufacturers of biologics utilizing porcine tissue or porcine tissue-derived materials, PCVs represent a contamination risk. In fact, an independent academic laboratory detected PCV-1 in a live attenuated rotavirus vaccine using metagenomic analysis and a PCV-1-specific polymerase chain reaction (PCR).[4-5] While this study did not detect PCV-1 or PCV-2 nucleic acid in rotavirus vaccine from a second manufacturer, subsequent testing by the manufacturer revealed low levels of both PCV-1 and PCV-2 DNA.[6] The source of the PCV nucleic acid contaminating both vaccines was determined to be porcine pancreas-derived trypsin used in the manufacture of the vaccines. The manufacturer of the rotavirus vaccine that was initially found to contain PCV sequences determined that their cell banks and virus seeds were contaminated with the viral sequences.[7-8] The strong safety record of both vaccines and the benefits of vaccination against rotavirus convinced both the United States Food and Drug Administration (US FDA) and the European Medicines Agency (EMA) to permit their continued use.[9-10]

In response to porcine trypsin as a source of PCV contamination, the EMA published a guideline for the use of porcine trypsin in biological products.[11] In addition to a number of risk mitigation measures, the guideline advises that two steps in the manufacture of porcine-derived trypsin be evaluated for their capacity to inactivate or remove small, resistant, non-enveloped viruses such as porcine parvovirus (PPV) and PCV. Evaluation of viral inactivation necessitates an infectivity assay (cell-based) over a nucleic acid assay, such as a quantitative PCR (qPCR) assay. Following an inactivation procedure, viral nucleic acid can remain and be detected by qPCR, even though no infectious virus is present. Traditionally, infectious PCVs have been detected using a cell-based assay with a fluorescent antibody or immunoperoxidase endpoint assay.[3] The low sensitivity of this assay results in low titers for PCV stocks, making demonstration of high levels of viral clearance problematic.

To provide increased assurance that infectious PCV would not enter the vaccine manufacturing processes, Merck & Co. (Kenilworth, New Jersey USA) and MilliporeSigma, a manufacturer of porcine-derived trypsin, partnered with BioReliance, to develop a quantitative infectivity assay for PCV-2. This assay enabled the evaluation of steps in the trypsin manufacturing process to inactivate PCV-2. During the manufacture of porcine-derived trypsin, an aqueous extraction at an ultra-low pH is performed for 12 to 24 hours. The exposure to very low pH for this duration has the potential to inactivate both enveloped and non-enveloped viruses. Historically, MilliporeSigma has demonstrated viral inactivation by the ultra-low pH extraction step utilizing xenotropic murine leukemia virus (XMuLV), bovine viral diarrhea virus (BVDV), pseudorabies virus (PRV), reovirus type 3 (REO-3), murine minute virus (MMV), and PPV, which are relevant or model viruses for a porcine tissue-derived product. Here we demonstrate the utility of a novel assay for evaluating viral clearance by assessing PCV-2 reduction during the ultra-low pH extraction step of the trypsin manufacturing process.

Methods and Materials

Preparation of PCV-2 Stock

PCV-2 obtained from the National Animal Disease Center (NADC; Ames, Iowa USA) was propagated in PCV-1 and PCV-2-free PK15 cells (also obtained from the NADC). The cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 0.1 mM non-essential amino acids in a humidified incubator at 37°C in the presence of 5% CO_2. PCV-2 was harvested 7 days following inoculation. The virus was concentrated by ultracentrifugation and suspended in a neutral buffer containing Tris, sodium chloride, and EDTA.

Immunofluorescence Assay for Infectious PCV-2

Virus titers were determined by immunofluorescence on PK15 cells that had been seeded into 96-well plates the day before inoculation. On the day of inoculation, serial dilutions of the virus were placed on subconfluent cells. Following a 7-day incubation, the cells underwent direct immunofluorescence staining using a fluorescent isothiocyanate (FITC) conjugated polyclonal anti-serum (VMRD, Inc.) that detected PCV-2. The anti-serum was shown not to react with other common porcine viruses. The number of positive wells at each dilution was used to calculate a 50% infectious dose ($TCIF_{50}$) value and 95% confidence limit using the Spearman Kärber method.[12]

New Infectivity Assay for Infectious PCV-2

PCV-2 was detected using an infectivity-based assay performed at BioReliance. Briefly, PK15 cells were seeded into 96-well plates the day before inoculation at a density that would yield subconfluent monolayers on the day of inoculation. On the day of inoculation, the culture medium was aspirated from the plates and serial dilutions of samples containing PCV-2 were applied to the cells. Each dilution of the sample was inoculated into eight replicate wells on each of two identical 96-well plates. The inoculated 96-well plates were placed in a humidified incubator at 37˚C in the presence of 5% CO_2. After 90 minutes, the plates were removed from the incubator, the inoculum removed from the wells, and the wells rinsed with fresh assay medium. The medium was removed and fresh assay medium was added to the plates and then returned to the incubator. One plate was harvested on day 0 for qPCR assay, and the remaining plate was incubated for 7 days post-inoculation and then harvested for assay. All wells were examined microscopically on days 0 and 7 post-inoculation. Harvesting was accomplished by aspirating the culture medium from each well and then storing the plates at or below –60°C.

The qPCR assay utilized the TaqMan® technology, using real-time PCR to amplify PCV-2 DNA. The detection of DNA sequences by PCR is a standard procedure where a specific fragment of DNA is amplified *in vitro* to generate many more copies of the original DNA fragment. Amplification for each sample and control in the assay was performed using forward and reverse oligonucleotide primers and a sequence-specific probe complimentary to sequences in the highly conserved ORF1 region of the PCV-2 genome. In the presence of the target sequence, the probe released a fluorescent signal that was detected on the ABI PRISM® 7900HT Fast Real-Time PCR instrument. The fluorescence data was then analyzed by the 7900HT sequence detection software. An exogenous internal positive control (IPC) was included to ensure that there was no inhibition of the PCR reaction.

Real-time PCR was performed on lysates prepared from cells in each well of the day 0 and day 7 plates, using primers and probe specific for PCV DNA. Samples which provided a cycle threshold value less than 40 ($C_T < 40$) were deemed positive for detection of PCV DNA, while samples which provided $C_T = 40$ (or undetermined) were deemed negative for PCV DNA detection. For each time point, no detectable fluorescence was observed in the negative control wells. The expected amplification was observed in the IPC wells of all plates analyzed from each time point, indicating that the test article, virus control, and negative control did not interfere with the PCR reaction.

The qPCR data from the day 0 and day 7 plates were used to quantitate replicating PCV-2 in a given well. Active infection was indicated by lower C_T mean values for a given dilution on the day 7 plate, as compared to the day 0 plate. Each well on the day 7 plate was scored as "positive" or "negative." Positive wells on the day 7 plate were identified as follows: The mean C_T value and standard deviation (SD) were calculated for each dilution from the day 0 plate. The C_T value for each well on the day 7 plate was compared to the mean minus 3 SD value for the corresponding dilution on the day 0 plate. If the value was less than the mean minus 3 SD, then the well was considered positive for infectious PCV-2. If the value was equal to or greater than the day 0 mean minus 3 SD, then the well was considered to be negative for infectious PCV-2. The number of positive wells on the day 7 plate at each dilution was used to calculate a 50% infectious dose (ID_{50}) value and 95% confidence limit using the Spearman Kärber method.[12]

Evaluation of Low pH Step to Inactivate PCV-2

The low pH step of the trypsin manufacturing process was evaluated for its capacity to inactivate PCV-2 according to the schematic shown in **Figure 1**. Low pH intermediate (MilliporeSigma) was clarified and then neutralized with 1 normal (N) sodium hydroxide. The neutralized intermediate

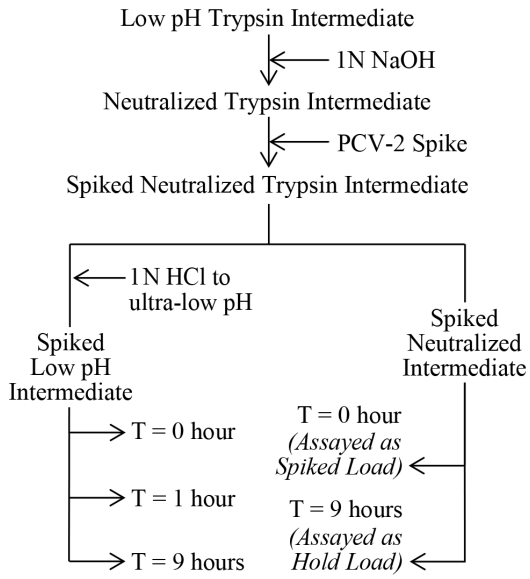

FIGURE 1. Schematic of the study to evaluate the inactivation of PCV-2 during incubation at ultra-low pH in trypsin intermediate.

was spiked to 10% with PCV-2 by BioReliance. An aliquot was removed to assay as the spiked load sample. A second aliquot of the spiked, neutralized material was held at the experimental temperature for the duration of the experiment (9 hours) and then assayed as the hold control.

Following removal of the load and hold control samples, the spiked intermediate was adjusted to the ultra-low pH using 1 N hydrochloric acid. A sample was removed for immediate assay (T = 0 hour, < 1 minute) and then at 1 hour and 9 hours.

The sample matrix, which contained myriad proteolytic enzymes, was potentially quite cytotoxic to the PK15 indicator cells. Thus, in order to reduce the cytotoxicity of the trypsin intermediate in the cell-based assay, a buffer exchange procedure was performed for all samples (**Figure 2**). Immediately upon collection, samples were diluted in a neutral pH buffer and then added to a Millipore Amicon Ultra-15, 50 kDa centrifugal filter unit. The retentate was washed twice with additional buffer. Following this procedure, an additional dilution was performed to bring the sample to the dilution that was previously shown to be non-cytotoxic in the virus detection assay.

Results and Discussion

Detection of PCV-2 by Infectivity Assay

The titers of PCV-2 stocks were determined by the new PCV-2 infectivity assay described above and a traditional infectivity assay with immunofluorescent antibody detection. The data shown in **Table 1** demonstrate that the new infectivity assay provided additional sensitivity, as evidenced by a titer that was over 1 log_{10} higher than the immunofluorescence assay.

PCV-2 Recovery Following Buffer Exchange

Since each sample would be subjected to buffer exchange, it was important to verify that no virus was lost during the procedure. In an initial experiment, PCV-2 was spiked into phosphate buffered saline (PBS) or the trypsin process intermediate and processed through the centrifugal filtration devices using the established procedure. PCV-2 genome copies were measured in the spiked load and retentate samples using a qPCR assay. The data in **Table 2** show that the total genome copies in the load and retentates were within 0.43 log_{10} of each other. This sensitive measure of viral nucleic acid demonstrates no loss of virus during the buffer exchange procedure. It also indicates that the trypsin intermediate sample matrix did not impact the recovery of the virus.

An additional experiment was performed to evaluate the recovery of infectious virus. PCV-2 was spiked into Hank's buffered salt solution (HBSS; spiked load) and processed through the established buffer exchange procedure. Total PCV-2 genome copies were measured by qPCR in the spiked load, the flowthrough, wash 1, wash 2, and the retentate (the actual sample that would be used in the infectivity assay). Infectious PCV-2 was measured in the spiked load and the retentate. Consistent with the results above, there was less than a 1 log_{10} decrease in genome copies (0.73 log_{10} copies) across buffer exchange. Additionally, there was less than a 0.5 log_{10} decrease in infectious PCV-2 (0.35 log_{10} ID_{50}) from the spiked load to the retentate, demonstrating that the buffer exchange procedure did not adversely impact the titers obtained in the PCV2 infectivity assay (**Table 3**).

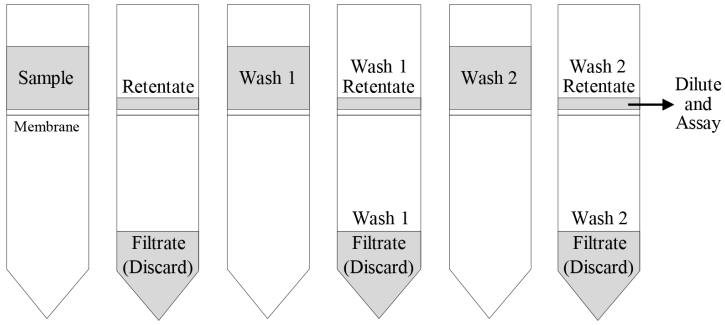

FIGURE 2. Schematic of the centrifugal filtration procedure used to exchange sample buffer for a less cytotoxic buffer.

TABLE 1. PCV-2 titers by immunofluorescence assay and the new infectivity assay.

Virus Stock	Log_{10} PCV-2 by Immunofluorescence Assay (Log_{10} $TCIF_{50}$/mL)	Log_{10} PCV-2 by Infectivity Assay (Log_{10} ID_{50}/mL)
PCV-2 Virus Stock*	5.28 ± 0.40	6.43 ± 0.35

**BioReliance lot number PC2120110X*

TABLE 2. PCV-2 DNA recovery following the buffer exchange procedure.

Matrix	Sample	Log_{10} PCV-2 Genome Copies (Log_{10} Copies)	Difference Between Spiked Load and Retentate
PBS	Spiked Load	10.91	0.10
	Retentate*	10.81	
Low pH trypsin intermediate	Spiked Load	10.52	0.43
	Retentate*	10.09	

**Retentate samples were processed through Amicon Ultra-15 50 kDa centrifugal filters.*

TABLE 3. Recovery of infectious PCV-2 from a spiked buffer solution following the buffer exchange procedure.

Sample	Infectious PCV-2 (Log_{10} ID_{50})	PCV-2 Genome Copies (Log_{10} Copies)
Spiked Load	5.27	11.27
Flowthrough	ND	6.38
Wash 1	ND	7.08
Wash 2	ND	7.13
Retentate	4.92	10.54
Difference between spiked load and retentate	0.35	0.73

ND = Not Done

Detection of PCV-2 in Trypsin Process Intermediate

Trypsin intermediate that had been through the buffer exchange procedure was evaluated for cytotoxicity and viral interference. To assess the cytotoxicity of the intermediate on the PK15 cells used in the PCV-2 assay, retentate from the buffer exchange procedure was serially diluted, and the lowest dilution that did not impact cell morphology (cause a cytopathic effect) was determined to be the non-cytotoxic dilution. The trypsin intermediate that had been processed through the buffer exchange procedure required a 1:30 dilution before cytotoxicity was eliminated.

The buffer-exchanged trypsin intermediate was also evaluated to determine whether or not any matrix component would interfere with the ability of the virus to infect the PK15 cells. Beginning with the non-cytotoxic, 1:30 dilution of the retentate, several serial dilutions were prepared. Each dilution was used as a diluent to assay a stock of PCV-2. In parallel, an assay was performed using buffer as the diluent. The lowest dilution of the retentate that resulted in a PCV-2 titer within 1 $\log_{10}$ of the titer generated in buffer was determined to have no viral interference. The 1:30 dilution of the retentate was determined to show no viral interference. The titer of PCV-2 in process intermediate was within 0.32 $\log_{10}$ of the titer determined in buffer (**Table 4**). Based on these cytotoxicity and viral interference data, all retentate samples were diluted 1:30 before they were used in the PCV-2 infectivity assay.

As another control, PCV-2 was spiked into neutralized trypsin intermediate or PBS at 10% (v/v) and assayed. The data in **Table 5** demonstrate that the trypsin sample matrix did not impact detection of the virus. Similar levels of virus were observed in both matrices: 5.95 $\log_{10}$ ID_{50} in neutralized trypsin intermediate and 6.04 $\log_{10}$ ID_{50} in PBS.

Cell-based viral infectivity assays can be impacted by the cytotoxicity of sample matrices, and use of these assays in viral clearance studies must include a careful evaluation of the cytotoxicity and viral interference of each process intermediate to be evaluated in the study. The trypsin process intermediate used in this study was a complex mixture of digestive enzymes with the potential to be very cytotoxic. The combination of a buffer exchange procedure and sample dilution successfully mitigated this cytotoxicity. The PCV-2 infectivity assay was shown to provide a robust measurement of infectious virus.

Low pH Inactivation

MilliporeSigma's routine trypsin manufacturing process contains an ultra-low pH hold for a minimum of 12 hours. In this study, inactivation at ultra-low pH for 9 hours was evaluated as a worst case for both pH and incubation time. The inactivation data are shown in **Table 6**. There was no decrease in PCV-2 infectivity in the neutralized trypsin intermediate over the 9-hour experiment: 5.95 $\log_{10}$ ID_{50} infectious PCV-2 was measured in the spiked load and 6.04 $\log_{10}$ ID_{50} in the spiked load hold. This indicated that the reduction in infectious virus in the ultra-low pH trypsin intermediate was pH-mediated, and not related to other attributes of the trypsin intermediate.

Once the neutralized trypsin intermediate was adjusted to the targeted ultra-low pH, a sample was immediately assessed for infectivity (T = 0 hr), but no decrease in infectious PCV-2 was observed. This was also the case at 1 hour, but by 9 hours, no infectious PCV2 was detected. To

TABLE 4. Evaluation of viral interference of trypsin process intermediates.

Sample	Total Infectious PCV-2 (Log_{10} ID_{50})	Difference between titer in trypsin intermediate and titer in buffer
PCV-2 diluted in buffer	5.88	0.32
PCV-2 diluted in 1:30 retentate	6.20	

TABLE 5. Detection of PCV-2 when spiked at 10% (v/v) in neutralized trypsin intermediate or PBS.

Sample	Total Infectious PCV-2 (Log_{10} ID_{50} ± 95% CL)
Spiked neutralized trypsin intermediate	5.95 ± 0.28
Spiked PBS	6.04 ± 0.33
CL = Confidence Limit	

TABLE 6. PCV-2 $\log_{10}$ reduction in ultra-low pH trypsin intermediate.

Sample	Total Infectious PCV-2 (Log_{10} ID_{50} ± 95% CL)
Spiked Load	5.95 ± 0.28
Hold Control (9 hr)	6.04 ± 0.33
PCV-2 reduction at 9 hr in neutralized sample matrix[a]	–0.09 ± 0.43
Spiked Load	5.95 ± 0.28
T = 0 hr	6.04 ± 0.32
T = 1 hr	5.77 ± 0.35
T = 9 hr[b] (titration)	≤ 3.07
T = 9 hr (large volume testing)	1.85 ± 0.86
Log_{10} PCV-2 reduction after 6 hr at low pH[c]	4.10 ± 0.90

a: *Log_{10} reduction in neutralized trypsin intermediate is difference in total virus between spiked load and hold control.*

b: *Infectious PCV-2 was not detected in the infectivity assay. Virus concentration was estimated using the Poisson distribution.*

c: *Log_{10} reduction calculated as the difference in total virus between spiked load and the T = 9 hr sample.*

increase the probability of detecting low levels of infectious virus, a larger sample volume from the 9-hour time point was evaluated for infectious PCV2. Only one of the 80 wells tested was positive for infectious PCV-2. The PCV-2 reduction at 9 hours was determined to be 4.10 ± 0.90 $\log_{10}$ ID_{50}.

These data indicate that PCV-2 infectivity decreases with increased time at ultra-low pH. MilliporeSigma's routine trypsin manufacturing process, which contains a targeted ultra-low pH extraction for a minimum of 12 hours, is capable of inactivating 4.10 $\log_{10}$ ID_{50} infectious PCV-2. These data support the capacity of the trypsin manufacturing process to inactivate any potential PCV-2 contaminants.

Conclusions

Animal-derived raw materials are required in the manufacture of many biopharmaceutical products. Prior to their use in a biopharmaceutical product, these animal-derived materials are extensively screened to ensure the absence of adventitious viral contaminants. Additionally, vaccines and other biological products and their intermediates must be tested for potentially contaminating adventitious viruses. However, due to inherent limitations in viral detection methodologies, the viral inactivation and/or removal through the raw material manufacturing process is often evaluated. As new testing methods are developed, such as sensitive non-specific, nucleic acid-based technologies, there is an increasing probability of revealing viral nucleic acid sequences in raw materials or biopharmaceutical products that were previously undetected. In fact, this type of testing revealed the presence of PCV nucleic acids in vaccine products.[4-5] Unlike an infectious virus, the presence of non-infectious viral nucleic acids does not pose a health risk. As nucleic acid detection capabilities become more sensitive, there is a need to develop robust and sensitive infectivity assays to be utilized in viral reduction studies.

PCVs do not produce reliable cytopathology in cells and historically, infectious virus was quantitated using immunofluorescence- or immunoperoxidase-based assays.[3] With this new infectivity assay, we have observed higher titers than with an antibody-based assay, facilitating demonstration of high levels of viral reduction. PCV-2 is closely related to PCV-1 and can serve as a surrogate for it in viral clearance studies. Demonstration of PCV-2 reduction by inactivation or removal steps in a manufacturing process can provide evidence of the capacity of these manufacturing steps to remove other closely related circoviruses.

The assay facilitates compliance with the EMA expectation for an evaluation of viral clearance for two orthogonal trypsin manufacturing steps by providing a means to quantitate this tiny, resistant virus by infectivity.[11] In addition to demonstrating the utility of this assay, we have also shown that the ultra-low pH extraction step of the trypsin manufacturing process can inactivate over 4 $\log_{10}$ of virus. This low pH extraction step can inactivate other resistant, non-enveloped viruses like PCV-2, adding to the safety profile for porcine trypsin and the biopharmaceutical products which utilize this animal-derived raw material.

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About the Authors

Tara Tagmyer, PhD, Merck & Co., Inc., West Point, Pennsylvania, USA; and
Kathryn Martin Remington, PhD*, Principal Scientist, Field Development Services
BioReliance, MilliporeSigma, 14920 Broschart Road, Rockville, Maryland 20850, USA
*Corresponding Author | Phone: 240-447-6280; Email: kathy.remington@sial.com