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Comparison of Extraction Methods to Detect Porcine Circovirus-2 and Porcine Reproductive and Respiratory Syndrome Virus in Semen Samples

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Abstract: Since, Porcine Circo Virus-2 (PCV2) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) are shed in semen for a long period of time after infection and artificial insemination is a common practice in pig farms, semen is an important source of spreading these viruses to naive populations. Therefore, rapid and accurate detection of PCV2 or PRRSV in semen could be critical at the standpoint of disease prevention and control. In this study, the performance of three different extraction methods on semen samples was compared to determine an optimal extraction method for semen samples to detect PCV2 or PRRSV. Seven or 115 semen samples were collected from the boars experimentally challenged with PRRSV or PCV2, respectively. These two sets of the semen samples were processed by three different extraction methods: High-Throughput Total Nucleic Acids Isolation kit (HT-TNA), High-Throughput Viral RNA Isolation kit with modified procedure (mHT-VR) and DNeasy kit or RNeasy kit for PCV2 or PRRSV, respectively. Then, the efficiency of the extraction methods were compared by conducting the same real-time PCR for each virus. HT-TNA and mHT-VR kits were faster and more convenient to process semen samples and HT-TNA kit showed higher or compatible sensitivity as compared to the other two methods while all three methods demonstrated 100% specificity. In conclusion, the HT-TNA Extraction Method could significantly reduce testing time and effort to process semen samples and showed better sensitivity for detection of PCV2 and PRRSV in semen.

Key words: Semen, high-throughput extraction, porcine circovirus-2, porcine reproductive, respiratory syndrome virus, swine

INTRODUCTION

Porcine Circovirus-2 (PCV2) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) are the two most important viral pathogens that cause serious damage to swine industry worldwide. These two viruses can spread fast among swine herds by horizontal transmission such as direct contact of ocular, nasal, fecal and urine discharge and by vertical transmission from infected dam to fetus (Andraud *et al*., 2009; Patterson and Opriessnig, 2010; Shen *et al*., 2010; Van der Linden *et al*., 2003). In addition since, both of the viruses can be shed in semen, semen should be tested for the presence of the viruses prior to Artificial Insemination (AI) (Krakowka *et al*., 2000; Opriessnig *et al*., 2007; Wasilk *et al*., 2004). The viruses present on both cell and seminal plasma fraction of semen but higher amounts of virus present in cell fraction than seminal plasma for both PCV2 and PRRSV (Pal *et al*., 2008; Wasilk *et al*., 2004). Because identification of the viruses in semen by conventional methods using tissue culture is very difficult due to the cytotoxicity of semen samples, PCR is routinely used to detect the viruses in semen. Therefore, optimization of both extraction and PCR procedures are important detecting viral DNA or RNA in semen samples. Currently, spin column-based extraction methods have been used more conventionally for molecular diagnostics. However, the column extraction methods may not appropriate for routine PCR testing to detect PCV2 and PRRSV from semen because it takes >4 h to complete nucleotide extraction from semen samples with the column-based methods and DNA for PCV2 or RNA for PRRSV should be extracted separately for the column methods (Cho *et al*., 2010). Moreover, cross-contamination between samples could be a problem in the column-base methods especially when many samples

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need to be handled simultaneously as reagents have to be manually added to and discarded from columns multiple times during extraction procedures. As compared to the column methods, magnetic bead-based extraction methods are much faster and more efficient since they can purify total nucleic acids (i.e., both DNA and RNA) from semen samples within 20 min and most steps in the extraction methods are performed automatically. In this study, three different extraction methods (Spin Column-Based or Magnetic Beads-Based Methods) were evaluated for their efficiency and accuracy in extracting viral DNA or RNA from semen samples.

MATERIALS AND METHODS

Preparation of semen samples: To prepare semen samples positive for PCV2, total 15 boars (12 boars were challenged with PCV2 and 3 boars were negative control) were selected for semen collection. The semen samples were collected at 6, 9, 13, 16, 20, 23, 55, 62, 69, 70 and 83 Days Post Inoculation (DPI) after challenge of PCV2. The raw semen samples from the experimentally infected boars were immediately aliquoted in 1.5 mL vials and stored at 80°C until DNA extraction. For PRRSV, 7 archived semen cell fractions stored in Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University was used. The semen samples were collected from either from 5 PRRSV-challenged boars and 2 PRRSVnaive boars. About 3mL of each raw semen sample was separated into cell and seminal plasma fractions by centrifugation at 800×g for 20 min and the cell pellet was resuspended in 2 mL of PBS and stored at 80°C.

Extraction of nucleotides from semen samples: The raw semen samples (for PCV2) or semen cell fractions (for PRRSV) were processed with three different extraction methods: MagMax™ High-Throughput Total Nucleic Acids Isolation kit (HT-TNA, Applied Biosystems, Austin, TX, USA), MagMax™ High-Throughput Viral RNA Isolation kit with HT-TNA protocol (mHT-VR, Applied Biosystems) and DNeasy or RNeasy kit (Qiagen, Valencia, CA, USA) for PCV2 or PRRSV, respectively as following procedures. For the HT-TNA Method, 300 μL of lysis solution was dispensed to a bead tube containing zirconia. Then, 200 μL of raw semen samples or semen cell fractions were added to the each tube containing the lysis solution. Each tube was sealed with silicon cap and beaten for 5 min with 20 Hz in TissueLysser (Qiagen). After bead beating procedure, the tubes were centrifuged at 1,6000×g for 6 min. 115 μL of the supernatant was transferred to a 96 deep-well microplate (VWR International, West Chester, PA, USA) containing 20 of paramagnetic beads and 65 μL of 100% isopropanol. For the automated extraction of nucleic acid in a 96 magnetic particle processor (Thermo Fisher Scientific, Waltham, MA, USA), one deep-well containing samples, two 96 well plates (VWR International) for washing Solution 1 and washing Solution 2 and one 96 plate for elusion were prepared. The 150 μL volumes of washing Solution 1 and washing Solution 2 and 50 μL of elution buffer were added on each well. The processing time was programmed by the manufacture's guide: 5 min for lysis/binding, 2.5 min for each washing step (i.e., washing 1 and 2), 1 min for dry and 3 min for elution. The extracted total nucleic acids in the elution plate were stored at -80°C until PCR reaction. The mHT-VR Method is a modified method which performs the HT-TNA protocol using MagMax™ High-throughput Viral RNA Isolation kit. In this procedure, 200 μL of raw semen samples or semen cell fractions were added to 300 μL lysis buffer mixture tube without zirconia bead. The tube was shaken for 5 min with 20 Hz in TissueLysser and remaining procedures were same as the HT-TNA Method.

Qiagen® 96 well Dneasy kit was used to extract PCV2 DNA (DNeasy Method) as described previously (Pal *et al*., 2008). About 200 μL of raw semen sample was added to a 1.5 mL microcentrifuge tube containing 20 μL of proteinase K and 400 μL of AL buffer and incubated at 65°C for 1.45 h. Then, 400 μL of ethanol (96-100%) was added to the mixture and mixed by vortexing. The mixture was transferred to the DNesy 96 column plate and centrifuged for 1 min at 4,000×g for the binding of DNA. The column was washed twice with AW1 and AW2 buffer and DNA was eluted with 50 μL of AE buffer. The DNA was kept at -80°C until PCR reaction. Lastly, Qiagen® RNeasy kit was used to extract PRRSV RNA (RNeasy Method) as described in a previous study (Christopher-Hennings *et al*., 2006). The 600 μL of RLT buffer was added a 1.5 mL microcentrifuge tube containing 200 μL of a semen cell fraction. The tube was vigorously vortexed for 15 sec and centrifuged at maximum speed for 5 min. The supernatant transferred to a new tube containing 600 μL of 70% ethanol and the total mixture was added to the RNeasy spin column as per manufacturer's instruction. RNA was eluted in 50 μL of RNase-free water.

Performance of real-time PCR or RT-PCR: As described previously, the extracts were subjected to real-time PCR (Opriessnig *et al*., 2003) or real-time RT-PCR (Kim *et al*., 2007) to determine the presence of PCV2 or PRRSV, respectively, using TaqMan chemistry. Real-time PCR was carried out by using Automated Real-Time PCR System (Applied Biosystems) with TaqMan® Fast Universal PCR

Master Mix (Applied Biosystems) in the 25 μL reaction volume while real-time RT-PCR was performed with QuantiTect® Probe RT-PCR kit (Qiagen) in a 25 μL reaction. Samples with a threshold cycle (Ct) ≤35 cycles were considered positive for both the PCR tests.

RESULTS AND DISCUSSION

Total, 115 semen samples were used to compare three extraction methods (HT-TNA, mHT-VR and Dneasy) for PCV2. Among 92 semen samples collected from 12 PCV2-challgend pigs, PCV2 was detected from 70 (76.1%), 40 (43.5%), 39 (42.4%) samples extracted by HT-TNA, mHT-VR and Dneasy Method, respectively (Table 1). PCV2 was not detected in 23 samples collected from 3 PCV2-negative pigs by any of the three extraction methods (100% specificity). The efficiency of PCV-2 detection was also evaluated by time (Fig. 1). PCV2 was detected from 50% of PCV2-challenged boars at 6 and 9 days after challenge (dpc), 75% at 13 dpc and 90% in average between 16 and 55 dpc when the HT-TNA Method was used for extraction whereas PCV-2 was detected only from 20 and 40% of the PCV2-challegned boars at 6 and 9 dpc, respectively and sensitivity never reached to 80% when either mHT-VR or RNeasy Method was used for extraction (Fig. 1). Seven semen cell fractions were used to compare three extractions methods (HT-TNA, mHT-VR and RNeasy) for PRRSV. Among 5 semen cell fractions prepared from PRRSV-challenged boars, only three cell fractions were positive by PRRSV Real-Time RT-PCR Reaction Regardless Extraction Method and Cycle threshold (Ct) of the real-time RT-PCR was not significantly different by extraction method, ranging from 29.59-33.65 Ct (Table 2).

PRRSV was not detected in the 2 samples collected from PRRSV-negative boars by any of the three extraction methods (100% specificity). These results indicated that the HT-TNA Method was more efficient in processing raw semen samples while all of the three extraction methods (HT-TNA, mHT-VR and Rneasy) were equally efficient in processing seminal cell fractions. It was speculated that the better efficiency of the HT-TNA in processing raw semen should be due to zirconia beads in the HT-TNA Method as the major difference of the HT-TNA Method from the other extraction methods was the use of zirconia beads which caused beneficial effects such as more vigorous disruption of semen samples and efficient removal of PCR inhibitory materials) as described previously (Cho *et al*., 2010).

Because the preparation of semen cell fraction from raw semen is quite laborious and time-consuming especially when many samples are involved and the amount of PCV2 in semen cell fraction is compatible to that of raw semen collected from PCV2-challenged boars (Pal *et al*., 2008), the direct use of raw semen for extraction shortens testing time without significant compromise of test sensitivity.

In addition, the HT-TNA and mHT-VR Methods in conjunction with the automated 96 magnetic particle

Table 1: Comparison of DNA extraction methods for the detection of PCV2 from raw semen samples

Extraction method	PCR result	PCV-2 challenge (n = 92)	Negative control (n = 23)	Sensitivity (%)	Specificity (%)
HT-TNA[a]	Positive	70	0	76.1	100
	Negative	22	23		
HT-VR[b]	Positive	40	0	43.5	100
	Negative	52	23		
DNeasy[c]	Positive	39	0	42.5	100
	Negative	53	23		

[a]MagMax™ High-Throughput Total Nucleic Acids Isolation kit; [b]MagMax™ High-Throughput Viral RNA Isolation kit with HT-TNA protocol; [c]Qiagen® DNeasy kit

Table 2: Comparison of RNA extraction methods for the detection of PRRSV from semen cell fractions

Animal ID	PRRS status	HT-TNA[a]	mHT-VR[b]	RNeasy[c]
203D14	Positive	30.39	29.59	30.20
203D20	Positive	32.26	31.58	31.87
203D28	Positive	33.19	33.26	32.52
395D28	Positive	N	N	N
585D7	Negative	N	N	N
585D20	Negative	N	N	N
651D28	Positive	N	N	N

[a]MagMax™ High-Throughput Total Nucleic Acids isolation kit; [b]MagMax™ High-Throughput Viral RNA Isolation kit with HT-TNA protocol and [c]Qiagen®RNeasy kit

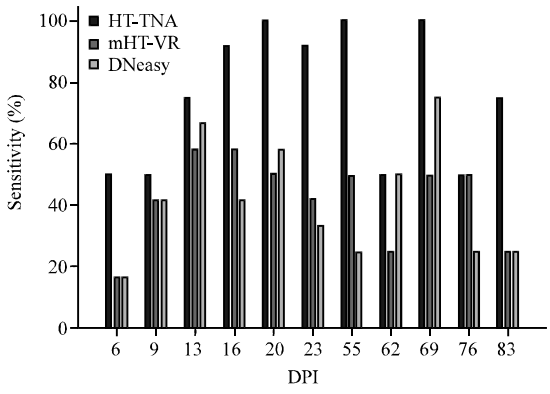

Fig. 1: Detection of PCV2 in raw semen samples sequentlly collected from 12 boars at 6-83 Days Post Infection (DPI) with PCV2. HT-TNA: MagMax™ High Throughput Total Nucleic Acids Isolation kit, mHT-VR: MagMax™ High Throughput Viral RNA Isolation kit with HT-TNA protocol and Dneasy: Qiagen® Dneasy kit

processor could process 96 semen samples for the extraction of both DNA and RNA within approximately 1 h while it took >5 h when DNeasy or RNeasy Method was used to process 96 samples.

Another advantage of using the automated system is reducing cross-contamination rate. Because conventional column-based extraction methods require multiple steps of adding several reagents manually, the chance of cross contamination between different samples is pretty high as compared to the automated Magnetic Beads-Based Extraction Methods.

CONCLUSION

HT-TNA increased the sensitivity in raw semen and remarkably reduced the sample processing time.

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