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 Circovirus-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 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 RNaseasy 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
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 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 experiment 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 3 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 DNaseq 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 each tube containing the lysis solution. Each tube was sealed with silicon cap and beaten for 5 min with 20 Hz in Tissuemizer (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 elution 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 manufacturer’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 Tissuemizer and remaining procedures were same as the HT-TNA Method.

Qiagen® 96 well Dneasy kit was used to extract PCV2 DNA (DNeasy Method) and 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 Dnaseq 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 elution was completed 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-Fleinnings 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 (Opriessig 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 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-challenged pigs, PCV2 was detected from 70 (76.1%), 40 (43.5%), and 59 (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 PCV2 was detected only from 20 and 40% of the PCV2-challenged 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>
<thead>
<tr>
<th>Extraction method</th>
<th>PCR result</th>
<th>PCV-2 challenge (n = 92)</th>
<th>Negative control (n = 23)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<td>43.5</td>
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<td></td>
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<td></td>
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<td>23</td>
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*MagMax™ High-Throughput Total Nucleic Acids Isolation kit; MagMax™ High-Throughput Viral RNA Isolation kit with HT-TNA protocol; Qiagen® DNeasy kit

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<th>Animal</th>
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<th>RNeasy</th>
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</table>

*MagMax™ High-Throughput Total Nucleic Acids isolation kit; MagMax™ High-Throughput Viral RNA Isolation kit with HT-TNA protocol and Qiagen® RNeasy kit

Fig. 1: Detection of PCV2 in raw semen samples sequentially 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 DNAeasy 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.

ACKNOWLEDGEMENTS

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