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# Effect of handling and storage conditions and stabilizing agent on the recovery of viral RNA from oral fluid of pigs



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## ABSTRACT

There is an increasing interest in using oral fluid to determine herd health and documenting the circulation of viruses in commercial swine populations but little is known about the stability of viruses in oral fluid. Hepatitis E virus (HEV) is a zoonotic virus which is widespread in swine herds. Information on optimal handling methods such as heat treatments, freezing and RNA stabilization agents is needed to prevent or minimize degradation of viral RNA by degradative enzymes. The objectives of the study were to determine optimum handling conditions of the oral fluid before RNA extraction and to compare the performance of the RNeasy Protect Saliva Mini kit, which contains a stabilizing agent, with that of the QIAamp Viral RNA Mini kit, which does not contain a stabilizing agent. Preliminary studies with oral fluid inoculated with HEV indicated that a heat treatment of 60 °C for 15 min was detrimental to HEV RNA. HEV was recovered from 25/25 and 24/25 samples of oral fluid when samples were incubated for  $\leq 24$  h at 4 °C and 30 days at –20 °C, respectively, without a stabilizing agent and extracted with the QiaAMP kit. In contrast, HEV RNA was detected in 16/25 and 11/25 samples when samples were incubated with a stabilizing agent for 24 h at 37 °C and 30 days at –20 °C, respectively, and extracted with the RNeasy Protect Saliva kit. Moreover, the mean number of genome copies/ml of HEV recovered from oral fluid stored at –20 °C without the stabilizing agent was 2.9 log units higher than oral fluid stored at –20 °C in the presence of the stabilizing agent. The recovery of RNA from HEV, F-RNA coliphage MS2 and murine norovirus (MNV), which are surrogates for norovirus, was significantly greater when oral fluid was incubated for 24 h at 4 °C than when oral fluid was stabilized with RNAprotect Saliva Reagent for 24 h at 37 °C, where the relative differences between the two processes were 1.4, 1.8, and 2.7 log genome copies/ml for MS2, MNV, and HEV, respectively. The findings suggest that it is unnecessary to stabilize oral fluid from swine for the detection of viral RNA, provided the samples are stored at 4 °C or frozen at –20 °C, and that the RNeasy Protect Saliva Mini kit did not perform well for the detection of viral RNA.

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## 1. Introduction

There is an increasing interest in detecting viruses in oral fluid. Oral fluid is defined as the fluid obtained from the mouth by an absorptive device (Atkinson et al., 1993). The composition of oral fluid is influenced by the location of the absorptive device in the mouth, the method of collection, as well as the collection device (Atkinson et al., 1993). Oral fluid is composed of mostly saliva from the salivary glands and serum transudate that crosses the oral mucosa and contains high levels of mucin, glycoproteins and enzymes such as amylases, proteases, lipases, RNAses (Prickett and Zimmerman, 2010). As viruses enter the oral cavity via serum, the tonsils, the upper and lower respiratory tracts, and environmental

contamination, PCR based assays have been used to detect nucleic acid from pathogenic viruses such as coronavirus, Ebola virus, hepatitis E virus, human herpes virus, and measles virus in oral fluids from humans (Collot et al., 2002; Formenty et al., 2006; Furuta et al., 2004; Matsubayashi et al., 2008; Wang et al., 2004) and swine pathogens such as foot-and-mouth disease virus, influenza A virus, porcine circovirus, porcine respiratory and reproductive syndrome virus (PRRSV), and Torque teno virus in oral fluid from pigs (Eblé et al., 2004; Prickett et al., 2008; Ramirez et al., 2012). Oral fluid sampling is considered an efficient and cost effective approach for surveillance of viruses in swine herds (Prickett and Zimmerman, 2010; Ramirez et al., 2012).

Hepatitis E virus (HEV) is a zoonotic virus and there are increasing reports of hepatitis E infections in humans in industrialized countries (Meng, 2011). HEV is widespread in swine herds but pigs are asymptomatic carriers or experience subclinical infections (Meng, 2011). Leblanc et al. (2007) reported that 98% of swine

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in a herd shed HEV in fecal material at some point between 2 and 29 weeks of age. HEV is transmitted through the fecal oral route (Bouwknegt et al., 2009; Kasorndorkbua et al., 2004) and pigs can be infected via direct contact with fecal material or by consuming feed or water contaminated with fecal material containing HEV (Leblanc et al., 2007). As pigs are imported and exported extensively, improved control for infected animals is recommended (Rutjes et al., 2007). While there is a lack of information on the prevalence of HEV in oral fluid from pigs, HEV is detectable in porcine tonsil tissue and salivary glands (Banks et al., 2004; Leblanc et al., 2010; Williams et al., 2001) at levels ranging from  $1 \times 10^3$  to  $9.9 \times 10^5$  genome copies (gc)/g of tissue (Leblanc et al., 2010). Furthermore, HEV RNA was detected in human saliva from an individual suffering from acute HEV where HEV RNA remained detectable in saliva, feces and serum until 71, 85 and 97 days, respectively, after transmission, suggesting the potential for the transmission of HEV also via the oral–oral route between humans (Matsubayashi et al., 2008).

There is an increasing appeal in using oral fluid to determine herd health and documenting the circulation of viruses in commercial swine populations (Ramirez et al., 2012). There are a number of advantages of detecting viruses in oral fluid over serum because of the ease of collecting the sample, the minimal discomfort of the subjects, it is non-invasive and cost effective. While the detection of viruses in oral fluid is an attractive possibility, there are concerns about the stability of viral RNA in the presence of salivary enzymes, proteins and cellular debris and information on optimal handling methods such as heat treatments, freezing and RNA stabilization agents is needed (Decorte et al., 2013; Roy et al., 1999). The objectives of the study were to determine optimum handling conditions of the oral fluid before RNA extraction and to compare the performance of the RNeasy Protect Saliva Mini kit, which contains a stabilizing agent, with that of the QIAamp Viral RNA Mini kit, which does not contain a stabilizing agent.

## 2. Materials and methods

### 2.1. Viruses

A swine fecal sample that was positive for HEV by qRT-PCR was diluted 1:5 in phosphate buffered saline (PBS) pH 7.4, incubated overnight at 4 °C and clarified by centrifugation at  $16,000 \times g$  for 5 min. The supernatant, which contained  $4.5 \times 10^6$  gc/ml of HEV, was used for inoculation experiments as a source of HEV. F-RNA coliphage MS2 (ATCC 15597-B1) obtained from American Type Culture Collection (ATCC, Manassas, USA) was propagated according to ISO 10705-1 protocol (Anon., 1995). Murine norovirus 1 (ATCC PTA-5935) was propagated in BV-2 cells, provided graciously by Dr. Mattison (Health Canada, Ottawa, Canada), as described by Cox et al. (2009). Stocks of MS2 and MNV were diluted in PBS to  $2 \times 10^6$  plaque forming units (pfu)/ml and  $4 \times 10^6$  pfu/ml, respectively, for inoculation. Feline calicivirus (FCV) was used as sample process control as described by Mattison et al. (2009).

### 2.2. Collection of oral fluid

Oral fluid samples were obtained from pigs (F1 sow Large white/Landrace bred to Duroc boars) reared in commercial barns at the Lacombe Research Centre. The barn has 28 pens and holds 15 pigs per pen. The average weight and age of the pigs were 105 kg and 150 days, respectively. HEV was not detected in fecal material of the herd after sampling repeatedly over a year. Oral fluid samples were obtained on sterile cotton wool swabs placed between two sterile rubber stoppers that were suspended from a sterile 1 m aluminum rod as described by Cook et al. (1997). A pig chewed on the rubber stoppers for approximately 2 min until the swab was saturated with oral fluid. Each sample of oral fluid was collected from

an individual pig in a pen, where only one sample was collected per pig. Each swab was placed into a sterile 15 ml tube containing a sterile 5 ml syringe, held on ice until centrifuged at  $1932 \times g$  for 20 min and the expelled oral fluid was transferred into a sterile 1.5 ml tube.

### 2.3. Treatment conditions and RNA extraction

For each of 25 oral fluid samples, 140  $\mu$ l was removed as a negative control while 655  $\mu$ l of oral fluid was inoculated with 165  $\mu$ l of clarified fecal HEV suspension and mixed by vortexing. The inoculated oral fluid was split into  $3 \times 140 \mu$ l portions and  $2 \times 200 \mu$ l portions. Each of the 140  $\mu$ l aliquots of oral fluid was (a) incubated for 1 h at 4 °C; (b) incubated for 24 h at 4 °C; or (c) incubated for 30 days at  $-20 \text{ }^\circ\text{C}$  while one of the 200  $\mu$ l aliquots was (d) incubated for 24 h at 37 °C or (e) incubated for 30 days at  $-20 \text{ }^\circ\text{C}$  (Table 1). Prior to RNA extractions, 2.5  $\mu$ l of  $1.0 \times 10^8$  pfu/ml of FCV was added to the negative control and each treated oral fluid sample. The QIAamp Viral RNA Mini kit (Qiagen, Mississauga, Canada) was used to extract RNA from the negative control and oral fluid subjected to treatments a, b, and c while the RNeasy Protect Saliva Mini kit (Qiagen) was used to extract RNA from oral fluid subjected to treatments d and e according to manufacturer's instructions. To support the findings obtained with HEV, 10 additional oral fluid samples were inoculated with diluted stocks of MS2 and MNV and were subjected to treatments b and d.

### 2.4. Detection by real time RT-PCR

HEV, MS2 and MNV were detected with the multiplex TaqMan RT-PCR assay for HEV, MS2 or MNV and FCV as described in Ward et al. (2009), Jones et al. (2009) and Brandsma et al. (2012), respectively, in 25  $\mu$ l of total volume using the Quantitect Multiplex no ROX RT-PCR kit (Qiagen). All primers and probes were synthesized by Integrated DNA Technologies (IDT, Coralville, USA) with the exception of the MNV ORF1/ORF2 probe that was synthesized by Applied Biosystems (Carlsbad, USA). Probes for HEV, MS2, MNV or FCV were labeled with 5'Cy5-3'Iowa Black RQ, 5'FAM-3'Black Hole Quencher (BHQ)-1, 5'FAM-3'Molecular Groove Binding Non-fluorescence Quencher, or 5'HEX-3'BHQ-1, respectively.

Each multiplex reaction contained 200 nM of each forward and reverse primers and probes, 0.25  $\mu$ l of Quantitect Multiplex RT mix, 0.03  $\mu$ M of ROX reference dye and 2.5  $\mu$ l of template in  $1 \times$  Quantitect Multiplex RT-PCR Master Mix. Thermal cycling conditions were one cycle at 50 °C for 20 min, then one cycle at 95 °C for 15 min followed by 45 cycles of 94 °C for 45 s and 60 °C for 75 s, performed on a Stratagene MX3005P QPCR thermocycler (Stratagene, La Jolla, USA).

DNA standard curves were constructed from serial 10-fold dilutions of  $10^6$  to  $10^0$  genome equivalents of purified DNA plasmids that contained the appropriate cDNA fragment in a 5 ng/ml salmon sperm DNA solution (Gibco BRL, Invitrogen Canada, Burlington, Canada).

### 2.5. Data analysis

The gc/RT-PCR assay was converted to log gc/ml of oral fluid using the calculation  $\log_{10}(\text{gc}/2.5 \mu\text{l (RNA extract)} \times 60 \mu\text{l (eluted volume)})/140 \mu\text{l (oral fluid)} \times 1000 \mu\text{l/ml}$ ) for RNA extracted with the QiaAmp kit and the calculation  $\log_{10}(\text{gc}/2.5 \mu\text{l (RNA extract)} \times 14 \mu\text{l (eluted volume)})/200 \mu\text{l (oral fluid)} \times 1000 \mu\text{l/ml}$ ) for RNA extracted with the RNeasy Protect Saliva Mini kit. Limits of detection HEV and MNV, based on limits of detection on the standard curve, were 3.2 log gc/ml for treatment a–c and 2.4 log gc/ml treatment d–e and limits of detection for MS2 were 2.2 log gc/ml and 1.4 log gc/ml for treatments a–c and treatments

**Table 1**  
Treatment conditions of oral fluid samples.

| Protocol | Treatment      | Volume of oral fluid ( $\mu$ l) | RNA extraction kit    | Elution volume ( $\mu$ l) |
|----------|----------------|---------------------------------|-----------------------|---------------------------|
| a        | 4°C, 1 h       | 140                             | QiaAmp                | 60                        |
| b        | 4°C, 24 h      | 140                             | QiaAmp                | 60                        |
| c        | –20°C, 30 days | 140                             | QiaAmp                | 60                        |
| d        | 37°C, 24 h     | 200                             | RNeasy Protect Saliva | 14                        |
| e        | –20°C, 30 days | 200                             | RNeasy Protect Saliva | 14                        |

d–e, respectively. For samples where the virus was not detected, the sample was assigned a number 1.5 log units below the limit of detection (Gill and Landers, 2003), which was 1.7 and 0.9 log gc/ml for samples extracted with the QIAamp Viral RNA Mini kit and RNeasy Protect Saliva Mini kit, respectively, to permit the comparison and separation of means. Mean values for each data set for each treatment of oral fluid inoculated with HEV were separated by the Tukey test of the general linear model procedure using Minitab version 14 statistical software (Minitab, State College, USA). A paired *t*-test (Minitab) was used to determine similarities for treatments of oral fluid samples inoculated with MS2 and MNV.

### 3. Results and discussion

There is an increasing interest in using oral fluid to determine herd health and documenting the circulation of viruses in commercial swine populations (Ramirez et al., 2012) but little is known about the stability of viruses in oral fluid. The few studies investigating the stability of RNA in oral fluid are related primarily to human mRNA as biomarkers of disease (Fábryová and Celec, 2013). Viral RNA can be present in oral fluid as intact RNA protected by the protein coat of the virus or accessible to RNases in the form of free RNA or associated with damaged virus particles (Roy et al., 1999).

The aim of the study was to evaluate different handling procedures to minimize the deterioration of viruses and viral RNA present in the oral fluid. Oral fluid contains degradative enzymes and some studies have suggested that a heat treatment may inactivate salivary enzymes and prevent degradation of viral RNA (Roy et al., 1999). Gomes-Kellar et al. (2006) incubated feline saliva samples at 42°C for 10 min before extracting nucleic acids and reported that there was good agreement with the detection of feline leukemia virus (FeLV) in saliva and plasma but average Ct values were 1.5 units higher in saliva. The effect of heat treatment on the performance of the assays was not investigated in that study but the initial levels of FeLV were fairly high in such samples, where mean Ct values were below 20. Roy et al. (1999) concluded that a heat treatment of 15 min at 60°C prior to storage at –20°C for 30 days did not prevent the degradation of hepatitis C virus (HCV) RNA in whole saliva and oral fluid samples during frozen storage but the effect of a heat treatment alone on the detection of HCV in those samples was not reported. Preliminary studies with oral fluid inoculated with low levels of HEV derived from a clarified fecal HEV suspension ( $5 \times 10^3$  gc/ml of oral fluid) indicated that a heat treatment of 60°C for 15 min was detrimental to HEV RNA (data not shown). While HEV was detected in 10/10 samples incubated at 4°C for 1 h and 18 h and in 3/10 samples stored at –20°C for 30 days, HEV was not detected in any of the samples that were heated for 15 min at 60°C or heat treated and then stored at –20°C for 30 days (data not shown). Based on those preliminary findings, the effect of heat treatment as a means of inactivating degradative enzymes was not investigated in subsequent trials.

Alternative methods to minimize or prevent the degradation of RNA in oral fluid include the addition of RNase inhibitors and

RNA stabilizers (Fábryová and Celec, 2013). Park et al. (2006) demonstrated that the RNA protect Saliva Reagent, included as part of the RNeasy Protect Saliva Mini kit (Qiagen), was significantly better than RNAlater or Superase Inhibitor in protecting salivary  $\beta$ -actin mRNA from degradation during storage at room temperature for 7 days, although the difference between RNA protect Saliva Reagent and Superase Inhibitor after 3 and 7 days of incubation was <1.3 Ct units. RNprotect Saliva Reagent can stabilize mRNA in saliva for up to 12 weeks, which permits the collection and transport of saliva samples at ambient temperatures and facilitates screening and diagnostics on a global level (Park et al., 2006).

There is no peer-reviewed information about the stability of viral RNA with the RNeasy Protect Saliva Mini kit, therefore the recovery of viral RNA stored at 4°C or –20°C and extracted with the QiaAMP Viral RNA Mini kit was compared with RNA stabilized at 37°C or –20°C and extracted with the RNeasy Protect Saliva Mini kit. The experimental conditions are representative of compatible storage conditions identified by the manufacturer. The recovery of HEV RNA without a stabilizing reagent was significantly greater than when RNA was stabilized with RNprotect Saliva Reagent (Table 2). HEV was recovered from 25/25 and 24/25 samples of oral fluid when samples were incubated for  $\leq$ 24 h at 4°C and 30 days at –20°C, respectively, without a stabilizing agent and extracted with the QiaAMP kit. In contrast, HEV RNA was detected in 16/25 and 11/25 samples when samples were incubated with a stabilizing agent for 24 h at 37°C and 30 d at –20°C, respectively, and extracted with the RNeasy Protect Saliva kit. Moreover, the mean number of gc/ml of HEV recovered from oral fluid stored at –20°C without the stabilizing agent was 2.9 log units higher than oral fluid stored at –20°C in the presence of the stabilizing agent. In all instances, the log number of gc recovered from each individual sample stored at –20°C was higher in the absence of the stabilizing agent (Table 2). The level of HEV inoculated into the oral fluid was >2 log units above the limit of detection.

HEV is extremely difficult to cultivate by cell culture (Berto et al., 2013). Therefore the source of HEV in this study was derived from a swine fecal sample that was positive for HEV by qRT-PCR. Other possible sources of HEV include serum or lymphnodes of infected animals, which were not available to the researchers. The fecal sample was diluted in PBS and clarified by centrifugation, a procedure used commonly for the detection of HEV by real time RT-PCR (Jones and Johns, 2012; Leblanc et al., 2007). One of the drawbacks of using fecal extracts is that fecal material contains inhibitors that may affect the PCR assay. The concentration of inhibitors originating from the fecal extract are diluted in the inoculated oral fluid where each sample of inoculated oral fluid contains approximately 3.3% of original fecal material, which may be realistic and comparable to the presence of background fecal material naturally present in oral fluid of pigs due to environmental contamination. Detecting viral RNA in oral fluid from pigs is complex. In addition to PCR inhibitors present in oral fluid itself, concerns have been raised about additional PCR inhibitors from fecal material and other environmental contaminants in oral fluid (Chittick et al., 2011). Chittick et al. (2011) reported that the interference of PCR inhibitors in oral

**Table 2**

Recovery (log genome copies) of hepatitis E virus/ml of inoculated oral fluid after incubation at 37 °C, 4 °C, or –20 °C and extracted with the QIAamp Viral RNA or RNeasy Protect Saliva Mini kit.

| Sample            | RNA stored without stabilization <sup>a</sup> |                  |                  | RNA stored with stabilization reagent <sup>b</sup> |                  |
|-------------------|---|------------------|------------------|--|------------------|
|                   | 4 °C (1 h)                                    | 4 °C (24 h)      | –20 °C (30 days) | 37 °C (24 h)                                       | –20 °C (30 days) |
| 1                 | 5.0   | 5.5              | 5.3              | <2.4   | <2.4             |
| 2                 | 4.9   | 5.3              | 4.6              | <2.4   | <2.4             |
| 3                 | 4.9   | 5.0              | 5.4              | <2.4   | <2.4             |
| 4                 | 4.8   | 5.1              | 5.1              | <2.4   | <2.4             |
| 5                 | 5.0   | 5.2              | <3.2             | <2.4   | <2.4             |
| 6                 | 5.2   | 5.5              | 4.6              | <2.4   | <2.4             |
| 7                 | 5.2   | 5.4              | 5.3              | <2.4   | <2.4             |
| 8                 | 5.5   | 5.7              | 4.7              | 4.5  | 3.6              |
| 9                 | 5.0   | 5.5              | 4.6              | 4.3  | <2.4             |
| 10                | 5.0   | 5.5              | 5.2              | 4.5  | <2.4             |
| 11                | 5.0   | 5.5              | 5.6              | 4.3  | <2.4             |
| 12                | 5.1   | 5.6              | 5.4              | 4.5  | <2.4             |
| 13                | 5.2   | 5.7              | 5.2              | 4.2  | <2.4             |
| 14                | 5.1   | 5.6              | 5.5              | 4.4  | <2.4             |
| 15                | 5.4   | 6.0              | 4.8              | 3.5  | 4.5              |
| 16                | 5.4   | 5.8              | 5.6              | 3.5  | 4.0              |
| 17                | 5.1   | 5.8              | 5.0              | 3.8  | 4.0              |
| 18                | 5.1   | 5.8              | 5.1              | 3.6  | 3.9              |
| 19                | 5.3   | 6.0              | 5.5              | 3.6  | 3.7              |
| 20                | 5.3   | 6.0              | 5.1              | 4.4  | 4.0              |
| 21                | 5.3   | 5.9              | 4.9              | 3.6  | <2.4             |
| 22                | 5.4   | 5.7              | 4.7              | 4.2  | 3.5              |
| 23                | 4.9   | 5.5              | 5.4              | <2.4   | 3.4              |
| 24                | 4.9   | 5.2              | 4.5              | 3.1  | 3.3              |
| 25                | 3.9   | 5.4              | 5.2              | <2.4   | 3.0              |
| Mean <sup>c</sup> | 5.1 <sup>A</sup>                              | 5.6 <sup>A</sup> | 5.0 <sup>A</sup> | 2.9 <sup>B</sup>                                   | 2.1 <sup>B</sup> |
| SD <sup>d</sup>   | 0.3   | 0.3              | 0.8              | 1.6  | 1.5              |

<sup>a</sup> Extracted with the QIAamp Viral RNA Mini kit.

<sup>b</sup> Extracted with the RNeasy Protect Saliva Mini kit.

<sup>c</sup> Means with the same subscript are not significantly different ( $P > 0.05$ ).

<sup>d</sup> SD – standard deviation, values below the limit of detection were assigned a value of 1.5 log units below the limit of detection.

fluid could be reduced by increasing the concentration of PCR enzymes.

The RNeasy Protect Saliva Mini kit did not perform well for the detection of HEV RNA compared to the QIAamp Viral RNA Mini kit, therefore the study was extended on a smaller scale to determine the relative recoveries of RNA from other viruses such as F-RNA coliphage MS2 and murine norovirus, both used as surrogate viruses for recovery and survival studies of norovirus (Brandsma et al., 2012; Jones et al., 2012; Park and Sobsey, 2011). MS2 and MNV were obtained from diluted virus stocks that were propagated by cell culture, which eliminates the concern about the presence of PCR inhibitors derived from clarified fecal extract as in the case of HEV. However, it is likely that the oral fluid still contains a background level of fecal material due to environmental contamination. Similar to results observed with HEV, the recovery of MS2 and MNV was significantly higher from oral samples that were incubated without a stabilizing agent but the relative differences in log genome copies recovered between the two processes were smaller than observed with HEV, with a difference of 1.4, 1.8, and 2.7 log units for MS2, MNV, and HEV, respectively (Table 3). While MS2 and MNV were spiked simultaneously in the samples of oral fluid, there was insufficient RNA for 2 of the oral samples to detect MNV by qRT-PCR for one of the extraction procedures, therefore those samples were excluded from the analysis for MNV. In contrast to HEV, MS2 and MNV were detected in all samples that were analyzed when oral fluid was incubated with a stabilizing agent for 24 h at 37 °C and extracted with the RNeasy Protect Saliva kit.

The RNeasy Protect Saliva kit has been evaluated for human mRNA but information on the performance with viral RNA or with oral fluid from animals is limited to a single final project report, which lacks peer review (Strugnell and Thirsk, 2010). The manufacturer instructions indicate that human RNA can be stabilized

with the RNA protect Saliva Reagent for up to 1 day at 37 °C, up to 14 days at room temperature, up to 28 days at temperatures between 2 and 8 °C, or archived at –20 °C or –80 °C, where lower temperatures are recommended when possible. The stabilization conditions of 24 h at 37 °C used in this study represent a worst case scenario within the guidelines of the manufacturer. Such conditions are encountered realistically by samples during collection in a warm climate or transit in vehicles. Although a better recovery of viral RNA might be expected when a lower stabilization temperature such as room temperature is used, the recovery of viral RNA was significantly lower from stabilized samples held at –20 °C than when oral fluid was extracted with the QiaAmp kit. However, even when PRRSV inoculated into oral fluid was stabilized with the RNA protect Saliva Reagent, serially diluted and then stored at 4 °C for 18 h before RNA was extracted, the performance of the QiaAmp kit was superior to the RNeasy Protect Saliva kit (Strugnell and Thirsk, 2010). The samples of oral fluid were inoculated with high levels of viruses in this study to permit the evaluation of different treatments in quantitative terms. However, when sampling oral fluid for viruses, investigators probably are more interested in presence/absence data than in quantitative data. The data suggests that at lower virus concentrations, the likelihood of detecting viruses is higher when the QiaAmp kit is used over the RNeasy Protect Saliva kit under the conditions that were tested. These findings confirm the observations of Strugnell and Thirsk (2010). In addition, stabilizing and extracting viral RNA with the RNeasy Protect Saliva Mini kit is almost 3 times more expensive than extracting RNA with the QiaAmp Viral Mini kit. As minimal degradation of viral RNA was observed with 3 different viruses when oral fluid was stored without a stabilizing agent at 4 °C for ≤24 h or –20 °C for 30 days and extracted with QiaAmp RNA Mini kit, it appears that it is unnecessary to stabilize oral fluid from swine for the detection of viral RNA, provided the samples are stored at 4 °C or frozen at –20 °C. The



**Table 3**  
Recovery (log genome copies) of F-RNA coliphage MS2 and murine norovirus (MNV)/ml of inoculated oral fluid after incubation at 4 °C or 37 °C for 24 h and extracted with the QIAamp Viral RNA or RNeasy Protect Saliva Mini kit.

| Sample            | MS2                                    |   | MNV                       |                                |
|-------------------|--|---|---------------------------|--------------------------------|
|                   | QIAamp Viral RNA Mini kit <sup>a</sup> | RNeasy Protect Saliva Mini kit <sup>b</sup> | QIAamp Viral RNA Mini kit | RNeasy Protect Saliva Mini kit |
| 1                 | 4.9                                    | 3.0   | 5.7                       | 4.1                            |
| 2                 | 4.8                                    | 3.9   | 5.6                       | 4.4                            |
| 3                 | 4.8                                    | 2.9   | 5.6                       | 3.5                            |
| 4                 | 5.0                                    | 3.2   | 5.8                       | 2.5                            |
| 5                 | 4.8                                    | 3.4   | 5.0                       | 3.8                            |
| 6                 | 5.0                                    | 3.7   | 5.8                       | 3.8                            |
| 7                 | 4.2                                    | 3.3   | 5.9                       | 4.2                            |
| 8                 | 4.8                                    | 3.3   | 5.6                       | 4.0                            |
| 9                 | 5.0                                    | 3.1   | – <sup>c</sup>            | –                              |
| 10                | 5.0                                    | 3.7   | –                         | –                              |
| Mean <sup>d</sup> | 4.8 <sup>A</sup>                       | 3.4 <sup>B</sup>                            | 5.6 <sup>A</sup>          | 3.8 <sup>B</sup>               |
| SD <sup>e</sup>   | 0.2                                    | 0.3   | 0.3                       | 0.6                            |

<sup>a</sup> Incubated for 4 °C for 24 h prior to extraction.

<sup>b</sup> Incubated for 37 °C for 24 h prior to extraction.

<sup>c</sup> –, insufficient volume of RNA for qRT-PCR assay.

<sup>d</sup> Means with the same subscript for each virus are not significantly different ( $P > 0.05$ ).

<sup>e</sup> SD – standard deviation.

findings of this study are in agreement with other studies investigating the stability of viral RNA in oral fluid from swine. Prickett et al. (2010) reported that PRRSV RNA in oral fluids was relatively resistant to degradation when samples were frozen promptly or held at 4 °C for up to 12 days. If refrigeration of samples of oral fluids is not possible, then it is important to use a stabilizer that works for viral RNA. Decorte et al. (2013) investigated the stability of PRRSV RNA in the presence of 3 different RNA stabilizers in pooled oral fluid at room temperature. PRRSV RNA was stable for 7 days at 4 °C without an RNA stabilizer and at room temperature with Oragene RNA as a stabilizing agent but when Aware Messenger or Saliva Gene Collection Module were used as RNA stabilizers, PRRSV RNA was degraded even more rapidly at room temperature than in the absence of these RNA stabilizers, with reductions of >3 log units after 8 h of incubation (Decorte et al., 2013). As the oral fluid samples were stored at –80 °C before the RNA was extracted, frozen storage may have had an additional impact on the results reported in that study. The findings obtained from the storage and handling conditions on the stability of HEV, MS2 and MNV in swine oral fluid and the limitations of the RNeasy Protect Saliva Mini kit contribute to the knowledge that is required to develop appropriate handling procedures to develop monitoring tools based on oral fluid collection to screen for the presence of HEV or other potential zoonotic viral pathogens in swine.

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