2012

Optimizing molecular detection and sequencing of Porcine Reproductive and Respiratory Syndrome virus in clinical oral fluid specimens

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Optimizing molecular detection and sequencing of Porcine Reproductive and Respiratory Syndrome virus in clinical oral fluid specimens

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Preventive Medicine

Program of Study Committee:

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Ames, Iowa

2012

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CHAPTER 1. REVIEW OF LITERATURE

Introduction

The Porcine Reproductive and Respiratory Virus (PRRSV, PRRS) is a problematic virus not only for pork producers but for the diagnostic labs that serve them. Detection of the virus by real-time reverse-transcriptase polymerase chain-reaction (real-time RT-PCR) has become widely used because of this assay's improved sensitivity and turnaround time compared to virus isolation. The purpose of this review is to evaluate the risk of missed PRRSV detection by real-time RT-PCR, with specific emphasis on a new sample type, swine oral fluids. The primary contributors to false-negative results include: virus mutation, viral degradation in clinical samples, and compounds that undermine the real-time reverse-transcriptase polymerase chain-reaction (real-time RT-PCR) chemistry, collectively known as 'PCR inhibitors'.

Importance of oral fluids as a diagnostic tool for animal health

A wide range of immune defense processes take place via the salivary proteins.[37] Advances in basic research of this sample type were gained during progression of human diagnostic testing for Human Immunodeficiency Virus (HIV).[43] Interestingly, HIV detection in oral fluid in combination with markers of disease also found in oral fluid, such as beta-2 microglobulins and/or tumor necrosis factor, were used together to monitor AIDS-disease status of patients.[37] As another example, detection of *Helicobacter* bacterial DNA from human oral fluid in combination with clinical signs has been used to identify infection in its clinically active state.[37]

There have been numerous reports of oral fluids in veterinary diagnostic roles in cats, dogs, cattle, and pigs.[43] Collection of swine oral fluids has proven to be easy as pigs naturally investigate and chew on new objects within their pen.[41] Samples are collected by hanging cotton rope shoulder-height in a clean area of the pen, allowing pigs 30 minutes to deposit oral fluid by chewing on the rope. Oral fluids are then extracted from the rope by squeezing the rope into a collection bag. Collection of this sample has the further advantage of maintaining biosecurity as it can be taken by resident barn personnel.[46] Modification of a commercially available PRRS ELISA has enabled its use in post-exposure measurement of PRRS antibody.[26] Sampling of a large number of animals and testing for a wide range of infectious agents is predicted to enable more efficient surveillance in the swine industry.[46]

The polymerase chain-reaction has rapidly become the preferred method of virus detection for food-animal production systems where timely results are needed. Specifically, fluorescence-based real-time RT-PCR has been transformed from an experimental technology into a mainstream scientific tool for detection of viral (and other) RNA. Qualitative detection (positive / negative) of viral RNA is possible as well as relative quantification of the level of virus in the sample; however, the sensitivity of detection and accuracy of quantification is dependent on many factors. Important issues such as sample collection, transport, sample processing, assay optimization, analysis and interpretation are important and sometimes overlooked.[8] Specifically for PRRS, current commercially available real-time RT-PCR reagents (LifeTechnologies, Inc. TaqMan NA and EU PRRSV reagents and Tetracore, Inc.

VetAlert NA and EU PRRSV PCR reagents) incorporate multiple primer and probes multiplexed to enable robust detection of variant PRRSV field strains.[20] This design allows amplification of the same template in multiple regions of the viral RNA, making its application as a PRRS quantification tool questionable compared to traditional single-target assay designs.

Until recently, swine serum has been the specimen type of choice for PRRS virus detection by real-time RT-PCR. Although serum remains the gold-standard, oral fluid has gained attention as a valuable alternative.[40-43,46] Under experimental conditions, pigs inoculated with PRRSV were shown to contain diagnostic levels of virus approximately four weeks after exposure enabling monitoring of disease by sampling in two to four week intervals.[41] A study in three commercial swine operations reported 77% agreement of PRRS real-time PRRS RT-PCR between serum and oral fluid samples.[40] Although oral fluid was slightly less sensitive in this study, a larger proportion of animals per pen is represented and more pen samples can be taken for the same cost making the overall approach preferable over serum collection.

Background

Under-appreciated role of PCR inhibition

While much work has focused on the strategic use of oral fluid for PRRSV detection, one aspect that may not be fully appreciated is the potential of this sample matrix to inhibit PCR reactions. PCR is an enzymatic reaction and therefore sensitive to inhibitors.[30,50] In a chapter on the subject in a recent PCR troubleshooting book, Gallup notes,

> "One of the least acknowledged problems with PCR, RT-PCR and qPCR is reaction inhibition."[19]

PCR inhibition often involves factors from the sample itself which interfere with or are not removed during the nucleic acid extraction process causing the final PCR assay to fall short of its potential of detection and quantification of its target. Prior to any other assay improvement, inhibition must be eliminated or the resulting cycle quantity (Cq, otherwise known as cycle threshold, Ct – the cycle at which a sample's fluorescent signal crosses an established threshold due to logorhthmic amplification) will not be proportional to the amount of template in the sample.[19] One technique to limit inhibition is to dilute the template. Gallup suggests the 'CRUD' rule: "**C**q values are only **R**eliable in **U**ninhibited qPCR reactions wherein high-quality samples and standards have undergone premeditatedly precise, dynamically sound **D**ilutions."[19] Known as the "sample effect"[19], each sample type (i.e. spleen, lung, serum, feces, oral fluid) has unique endogenous inhibitors.

> "Only after crud is not allowed to interfere with the RT and PCR reactions can we accept the entire process to be capable of yielding quantitatively informative or biologically relevant information,"[19] says Gallup.

The exact sources of PCR inhibition within swine oral fluid have not been fully defined but neutralization and/or elimination of these sources remain an important step in the development of efficient pre-PCR treatment.[17]

Swine oral fluid PCR inhibition demonstrated

Initially, detection of PRRSV in swine oral fluid by PCR was found to be inadequately sensitive using methods designed for serum. Stensland and Kim of the

Iowa State University Veterinary Diagnostic Lab (ISUVDL) developed a modified method optimizing sample lysis buffer and more vigorous agitation of samples during lysis [unpublished data]. In a later study, evaluation of internal controls showed that 4.6% (2/43) of internal controls still failed using this method (method A1, magnetic bead-based, viral RNA extraction kit with modified lysis buffer and increased lysis step intensity).[10] These potential false-negatives were significantly reduced when the enzyme mix (taq polymerase and reverse-transcriptase mixture) concentration was optimized by, in this case, doubling the quantity of enzyme in the mastermix.

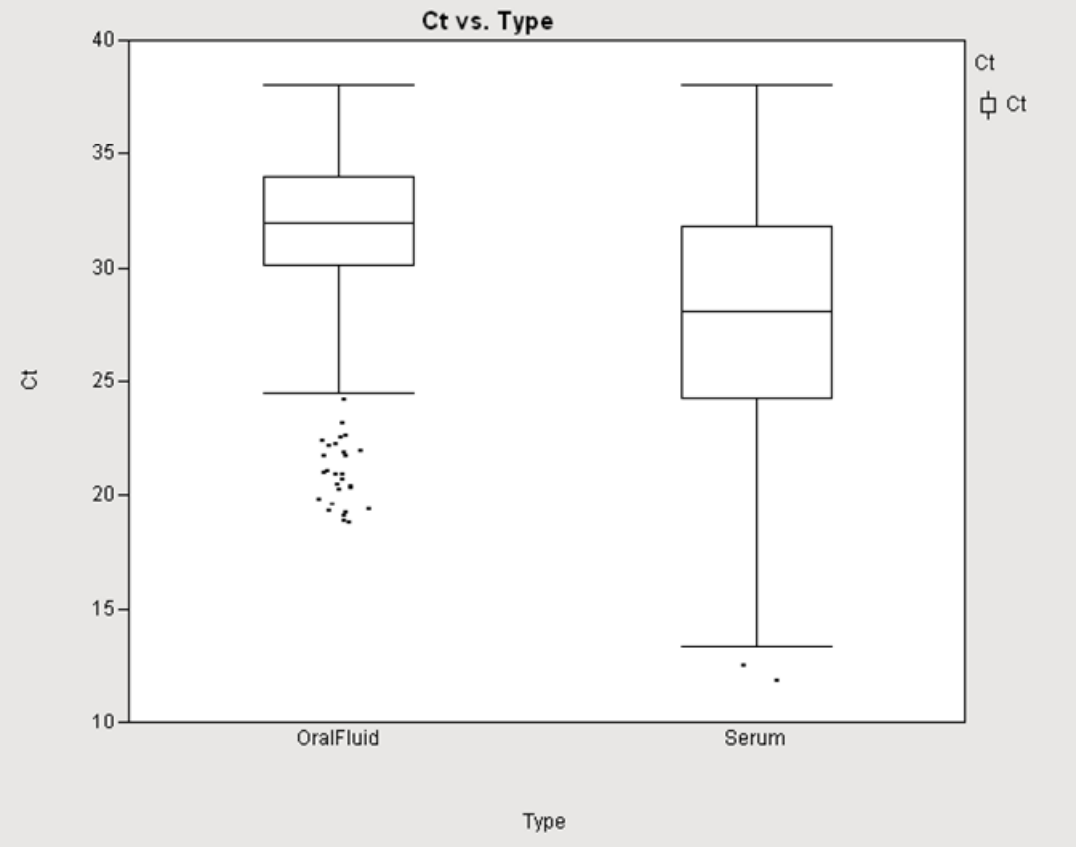
The method that had the best sensitivity in this study involved increasing sample input volume along with larger volumes of RNA purification reagents in the extraction process (method A2). Interestingly, sensitivity improvement using the larger-volume extraction method was only revealed after increasing the enzyme concentration in the PCR reaction.

In summary, large-volume extraction followed by increased PCR enzyme concentration in the reaction resulted in a significant improvement in detection of PRRSV, eliminated failed internal controls (in study samples), and significantly reduced variation of internal controls in the study samples.[10] This observation is consistent with reports that inhibition of Taq and RT enzymes used in PCR is the most common cause of overall PCR inhibition.[57]

The best PRRS PCR extraction and PCR method from this study[10] was implemented in routine diagnostic testing of swine oral fluid submissions received at the Boehringer Ingelheim Health Management Center Diagnostic Lab. Serum samples were tested using extraction and PCR methods as described elsewhere.[20]

The distribution of Cq values from real-time PRRS PCR positive oral fluid and serum field samples from the same time period is presented in Figure 1. Differences in Cq data between specimen types is shown to highlight differences in PRRS virus detection and concentration between these two sample types as they are currently tested, discussed further in following sections.

Figure 1: PRRS real-time PCR Cq distribution from RT-PCR-positive oral fluid and serum diagnostic samples tested between September 1, 2011 and August 31st 2012 at the Boehringer Ingelheim Vetmedica Health Management Center Diagnostic Lab.



	Oral Fluid	Serum
Number of Positive Samples (n)	2381	4854
Mean Cq	32.1	28.1
Minimum Cq Observed	18.7	11.8
Maximum Cq Observed	38 (assay cutoff)	38 (assay cutoff)
Percent (failed/total) of Failed Internal Controls among All Samples Tested	1.7% (124/7205)	0.2% (48/24030)

Challenges to PRRSV RT-PCR diagnostic sensitivity

Primer/probe miss-matches to mutating virus

Although RT-PCR is theoretically sensitive enough to detect one PRRS virion in a sample under ideal conditions, a number of factors may prevent this from occurring in the dynamic situation faced by diagnostic laboratories and veterinarians chasing ever-changing field strains. As a single-stranded, positive-sense RNA virus with a 15kb genome, PRRS mutates at a relatively high rate as it is transmitted from pig to pig over time.[33] RNA viruses are inherently more prone to mutation, due in part to the infidelity of the RNA polymerase it uses to replicate. The calculated rate of PRRSV nucleotide substitution is the highest reported so far for an RNA virus, estimated as 4.7-9.8 x 10-2 / nucleic acid site / year.[33]

Detection of virus in clinical samples by real-time RT-PCR works best when the viral RNA target is conserved and stable. Hydrolysis probe (Taq-Man®) real-time PCR chemistry functions by making copies of a specific amplicon as a result of primers binding to the target, followed by fluorescent signal generation if the amplicon-specific probe hybridizes to its complimentary target.[9] Current commercial PCR assays (Tetracore Inc. and Life Technologies) for the mutating PRRS virus utilize multiple primers and probes in a multiplexed PCR assay format as a strategy to ensure adequate detection.[20] In spite of this strategy, in a recent study PRRSV was missed in 6% (27 of 423) of the diagnostic cases evaluated (of multiple different sample types including lung, serum, oral fluids, and others) when reliance was placed on a single real-time PRRS PCR test alone.[20]

The effect of sample pooling

Pooling of samples, a common practice, is a significant risk factor in missed detection[20], and has previously been proven to reduce sensitivity.[49] In serum and blood swab samples, pooling 1:3 and 1:5 decreased sensitivity over the course of the study by 6% and 8% respectively with an even more pronounced effect within the first five days post-infection, with up to 14% missed-detection in pooled samples.[49] As shown in figure 1, serum diagnostic samples contain lower Cq values (and thereby higher concentration of virus) than oral fluid samples. It would be expected, based on concentration alone, that pooling of oral fluid samples may present an even higher risk of missed detection by overly diluting the target beyond the detection limits of the test.

Sample integrity / degradation

RNA is universally understood to be sensitive to degradation due to many contributing factors even in controlled conditions.[34] An assay for measuring mRNA has been developed to determine if degradation of target RNA occurred within a sample rendering it unsuitable for testing.[34] Viral RNA was tested alongside mRNA and it was concluded that viral RNA degradation contributes to false-negative PCR reactions.[34] Bustin refers to DNA as “tough as old boots” and RNA as extremely delicate once removed from the environment.[8] Detection of RNA viruses from oral fluids has been noted as more complicated because single-stranded RNA is much less stable than double-stranded DNA, in this specimen type[37] leading to degradation prior to testing. Despite of the labile nature of pure RNA, viral RNA within an intact PRRS virion is expected to be more stable according to two studies

completed on the subject.[24,42] The issue of PRRS virus stability in swine oral fluids is important due to the relatively lower viral load of swine oral fluids versus serum (see figure 1). Virus concentration falling below the limit of detection of the real-time RT-PCR as a result of degradation presents another risk of false-negative results.

Stability of the PRRS virus in buffered media as measured by virus titration and quantitative PCR has been reported. Infectivity of virus was shown to decline quickly as a function of time and temperature as measured by cell culture. Under the conditions of this study, the authors concluded that viral RNA was stable at all times and temperatures evaluated[24]; however, the concentration of virus used was much higher than what is commonly seen in field samples and warrants further study using levels of virus more indicative of clinical samples. Further, viral degradation in this study was evaluated in virus-friendly buffered media; stability would be expected worse in contaminated, complex sample matrices. For this reason the conclusion of stability may not be directly applicable to clinical swine oral fluid samples.

Stability of PRRS virus in the oral fluid sample matrix has been specifically examined.[42] A pool of oral fluids collected from 16 week old finisher pigs was spiked with virus for a final concentration of 10^9 copies / ml and sampled over a 12 hour period during which treatments with antimicrobial preservatives occurred. Relative to virus load in diagnostic samples (see figure 1) this concentration is extremely high corresponding to a Ct value of about 11 (assuming 1 copy detection limit at Ct of 38, with 3 Ct interval for every log of virus change). By comparison, this level of PRRS virus spiked into oral fluid is 10,000,000-fold more concentrated than the mean field sample (Ct of 32 ~10^2 copies) and 10^9 copies of PRRS virus is 1000-

fold higher than the highest observed field sample (Ct of 19 ~ 10^6 copies). The conclusions of the authors were that temperature had a significant effect on virus stability but refrigeration of samples "will maintain integrity of PRRSV RNA for diagnostic testing."[42] In spite of this conclusion, mean quantity of immediately-frozen samples were $10^{7.8}$ $\log_{10}$ genomic copies per mL where means of refrigerated sampling was $10^{7.3}$ $\log_{10}$ genomic copies per mL; a loss of 4.31×10^7 genomic copies per mL, apparently due to degradation at refrigerated temperatures. Though not reported as statistically significant, this level of virus loss is troubling. Given the fact that mean virus load found in oral fluid field samples average around 100 to 1000 copies detected (estimated based on mean Ct 32 of 2381 samples tested over 12 month period, figure 1), a loss of the published magnitude could apparently lead to a positive sample testing negative due to degradation during refrigeration.

PCR Inhibition

In addition to each of these issues impacting diagnostic sensitivity of the PRRS virus, inhibition of PCR presents an important hazard, particularly in swine oral fluid and feces. In studies of Hepatitis A in human saliva it was noted the major limiting factor in amplification reactions using oral fluids are low target RNA concentration and the presence of inhibitory substances.[3] Testing for the Epstein-Barr virus in humans, oral fluids were shown to 'exert a potent inhibitory effect on PCR'.[35] A number of studies and review papers have looked at PCR inhibition and provide overviews of mechanisms, targets, and sources of PCR inhibition.[,19,29,31,48,50,57] In Bluetongue virus research among camelids, Brito notes,

> "Unfortunately, PCR inhibitors have not been critically studied or defined in reality for most animal species despite the broad use of PCR in veterinary diagnostics."[6]

Use of an internal control (also known as a 'mimic' or 'competitor RNA/DNA') has been shown repeatedly to be extremely important in detection of false-negative reactions.[27,2,8] A mimic shares the priming region of the assay's target primers but contains an insert of non-native DNA or RNA.[27] An exogenous internal amplification control consists of nucleotide sequence that is uncommon to any part of the target, requiring separate primers and probe for detection is a preferred method to avoid reporting false-negative results.[8] This control is now a part of commercially available PRRSV RT-PCR reagent sets and has been used to detect false negatives and highlight increased variability associated with PCR inhibited samples.[10] As the use of the PRRS real-time PCR test method moves from secondary confirmation of clinical cases to use as a tool for surveillance associated with eradication, an increased scrutiny on internal controls is needed.

Sources of inhibition

Components / molecules

Most known inhibitors are organic, examples include: bile salts, urea, phenol, ethanol, polysaccharides, humic acids, melanin, as well as different proteins such as haemoglobin, immunoglobin G and proteinases. Divalent cations including calcium and iron are problematic as well as they interfere with the activity of magnesium in the PCR reaction. Interference with fluorescent probes used in real-time PCR can increase background, thereby decreasing sensitivity. Many inhibitors affect RNA

directly.[50] An automated high-throughput filtration assay has been reported as a way to characterize the actual polymerase inhibitor compounds in samples, it may be a valuable tool for inhibitor characterization of swine oral fluid.[58]

Cellular debris remaining after total nucleic acid extraction has been implicated in RT and PCR inhibition by altering enzyme kinetics and associated Cq acquisition.[54] Blocking DNA from PCR priming by polysaccharides or proteins endogenous to the sample have been implicated.[54]

Excess RNA or DNA can itself be an inhibitor, such as in flora-dense samples. Swine oral fluid samples may be considered 'flora-dense' as previous work has shown high levels of bacteria present.[42] Human saliva has been found to contain large amounts of RNA. Human saliva was also found to contain mucins which may have a role in antigen capture or PCR inhibition.[36] Mucin, a component of oral fluid, has been shown to inhibit PCR when added directly to mastermix, inhibiting some forms of Taq polymerase more than others.[1]

Sample types

Each host animal species and sample matrix have unique properties requiring separate characterization of their inhibitory properties.[6] For example, human feces have been found to completely inhibit PCR in dilutions as high as 1:1000, potentially due to bile salts.[30] Fecal samples are a highly variable sample matrix dependant on nutrition and gut flora making inhibitor characterization and generalization difficult.[50]

A range of bovine sample types have been found to contain varying levels of contaminants affecting performance of mRNA qPCR. Among sources evaluated,

tissue type (among cerebellum, liver and muscle) was noted as having the largest source of variance compared to influence of target gene.[54]

PCR inhibitors from food samples have been noted as early as 1992.[48] Several different inhibitors have been detected in animal feed.[31,50] One author has gone so far as to say background flora and presence of inhibitors in feed make it an unsuitable sample type for PCR.[31] This is an important finding as feed is commonly found in swine oral fluid samples.

Whole blood is known to be an inhibitor of PCR due to the heme portion of the red blood cell.[12,50] Bone samples have been found inhibitory to PCR in forensics studies, making detection of low concentration target DNA difficult.[16] Relatively low concentration of target is similar in PRRS detection from swine oral fluids (see figure 1).

Laboratory-derived inhibitors of PCR can include materials used in extraction purification such as ethanol, isopropanol, phenol, and even powder from disposable gloves if not appropriately controlled.[50] Preservatives in plasma separation tubes have been found to be both inhibitory to PCR as well as a source of over-estimation of HIV type 1 virus.[28]

Impacts of PCR inhibition

False negative PCR due to inhibition has real impacts on veterinary diagnostics and the clients they serve. In an assessment of a variety of serum samples (n=470) prepared from routine submissions at the University of Missouri VDL, partial or complete inhibition of the real-time RT-PCR was found in 20% of the samples where 14% were totally inhibited and 6.6% were partially inhibited. This

was detected by spiking competitor RNA into extracts at a 10-fold greater concentration than the detection limit of the PRRS RT-PCR assay.[27]

In a study of *Salmonella* detection in feces from human clinical cases, 8% of samples (n=120) were inhibited (false-negative), including two culture-positive samples.[2] Ante-mortem diagnosis of *Lawsonia* in swine requires PCR testing of feces, although 25 of 60 fecal samples tested in one study were found to be inhibited.[25] Several studies on the presence of *Lawsonia* in feces discuss low / intermittent shedding as a major challenge of diagnosis by fecal PCR. After controlling for inhibition through the use of a mimic internal control, applicability of this test method may lead to different conclusions of the PCR test utility and the pathogenesis of the organism.[25]

Avian feces are notorious for PCR inhibitors that are difficult to remove.[5,13] In spite of using modern magnetic-bead capture extraction methods, 18% of 2668 clinical samples studied had evidence of inhibitors that may have prevented detection of Avian Influenza by RT-PCR.[13] Detection of Helicobacter in human feces has been impacted by inhibitory effects of bile, leading to discrepant research findings that were likely due to the presence of PCR inhibitory substances.[1]

Intervention Strategies

Processing

Pre-treatment of specimens before nucleic acid extraction has been evaluated with some success. In some cases enrichment by culture of bacteria or virus from a clinical sample may improve PCR sensitivity to overcome the effect of inhibition, although components of the enrichment media itself may prove to be

inhibitory if not removed. When accompanied with extraction aimed at removal of inhibitors, sensitivity to *Salmonella* from human feces was improved to the extent that a nested PCR could be replaced with a single-reaction PCR.[2]

Extraction

Bustin, et al has indicated that most problems in reproducibility of RT-PCR stem from sample collection and purification of RNA template.[8] Compounds inhibitory to PCR can become concentrated by the extraction method by co-precipitation with the nucleic acids.[19] Purification of sample RNA or DNA is the purpose of 'extraction' performed prior to PCR testing, but differentiating improvements due to reduction of inhibitory components vs. increased efficiencies of extraction of the actual target is not trivial. Separating these differences has been noted as a confounder of a number of early comparison studies and an alternate statistical analysis was proposed to control for it.[17] Modification of the viral lysis step (including more vigorous mixing and modified lysis buffer composition) has proven important for efficient extraction of viral nucleic acids from both swine and avian samples.[10,13]

Use of magnetic-bead capture has proven to be a significant advancement in removal of a wide range of inhibitors.[10,13,50] Improvements in sensitivity and removal of inhibitors by bead-based systems over silica-gel (spin-column) based systems have been noted in a number of studies.[10,13,50] In some instances further optimization of magnetic-bead capture systems was still required to address unacceptable levels of false-negatives.[10,13,50]

PCR

Appropriate design of primers has been shown to improve an assay's ability to overcome or resist PCR inhibition. Target sequences that amplify with a higher efficiency better resist inhibition, making documentation of PCR efficiency important in assay design. [7,50,54]

Use of the appropriate Taq and/or Reverse-Transcriptase enzymes have been found to improve resistance to inhibition.[16,23] A thorough review of classification of types of PCR polymerases and their functionality was discussed elsewhere in an attempt to address PCR inhibition from bone samples.[16] Briefly, DNA polymerases fall into distinct categories, including seven categories of which the most extensively utilized is *Taq* DNA polymerase. The enzymatic properties such as nuclease activity, fidelity, extension rates, and processivity (the number of nucleotides added before disassociation of the enzyme) vary widely among families of polymerases. Evaluation and consideration of the most appropriate polymerase can be a productive way to reduce or eliminate PCR inhibition. [16]

Dilution of nucleic acids prior to PCR has been shown effective as it is thought to increase the physical distance between the inhibitors and the target molecule.[1] It has been described, somewhat counter intuitively, that adding more sample to the PCR reaction (RNA extract in this case) does not always decrease Cq of the reaction when inhibitors are present in the sample. Any nucleic acid sample subjected to RT-PCR, PCR, or qPCR can harbor varying degrees of inhibitory material; therefore the practice of using one randomly chosen blanket nucleic acid dilution for all targets is not advisable. For each specimen source, the 'inhibitory

characteristic', or appropriate dilution level, must first be determined. This approach finds the optimum trade-off between dilution of template and dilution of inhibitors.[19]

Conclusion

PRRS real-time RT-PCR plays an important role in swine herd control and elimination efforts of producers to eliminate PRRS virus from their systems.[15,49] One metric used to measure efficacy of intervention strategies is 'Time-To-Negative', defined as the time it takes post- herd intervention to produce PRRS-negative groups of weaned pigs.[38,39] In these types of studies, false-negative results from any source complicate progress and potentially undo months of work to eliminate the virus if positive animals are allowed to remain in the herd.[38,39]

The Pork industry has continued to do battle with the PRRS virus and efforts are underway to control and eliminate the virus through coordinated Area Regional Control and Elimination projects. Large scale PRRS control and elimination are estimated to cost $560 million dollars annually and may take multiple years.[39] As this effort relies heavily on accurate detection of the PRRS virus, diagnosticians and veterinarians alike need to understand the limitations and assay maintenance required to keeps the real-time PRRS RT-PCR methods current.. As oral fluids are evaluated for their role in monitoring or elimination programs, stability during transport needs further investigation and optimization. Internal controls must be integrated into testing protocols for this sample type to ensure false-negatives results are prevented. False-negative PCR results, while often less frequently discussed than false-positives, have serious consequences when they occur. In a

letter to the editor of the Journal of Clinical Microbiology, a concerned diagnostician writes

> "It is the false-negative results that turn a risk into a threat for the population, whereas a false-positive result merely leads to a clarification of the presumptive results by retesting the sample."[22]

In both human and animal research there is a need for internationally recognized standards in reporting PCR assays to ensure adequate method validation[7] including ring-trial validation and mandatory use of internal amplification controls.[22] Thorough reporting of all aspects of PCR in scientific reports is lacking and must be improved in order to make efficient progress over time with research dollars.[7]

It is required by ISO 17025 laboratory accreditation that each test method be validated for each specific sample type tested before accreditation for that test/sample type is granted, which reflects an underlying principle in test validation. In assessing the difficulties of detection of Salmonella in feed samples, Lofstrom wrote,

> "When optimizing PCR-based detection, the intended samples should be considered from the beginning because the sample itself affects the biochemical composition of the PCR mixture and thus the outcome of the analysis."[31]

In conclusion, accurate diagnosis of the PRRS virus by real-time RT-PCR has continued to prove effective in the majority of cases; however a number of challenges are faced daily by diagnosticians and researchers. The goals of the following research were to optimize and improve molecular test methods for PRRS detection in swine oral fluids in support of the potential this sample matrix promises the swine industry.

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CHAPTER 2. COMPARISON OF ORAL FLUID PRRS PCR METHODS

A paper published in the *Journal of Veterinary Diagnostic Investigation*

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Abstract: The objective of the current study was to evaluate various RNA extraction and polymerase chain reaction (PCR) protocols for the detection of *Porcine reproductive and respiratory syndrome virus* (PRRSV) in porcine oral fluids. Extraction protocols were selected based on ease of use and compatibility with high-throughput, automated systems. The results showed marked differences among extraction protocols, PCR protocols, and combinations thereof in detecting PRRSV in the oral fluid matrix. An important finding was that PCR reactions were partially inhibited by unknown factors in the oral fluid matrix and that inhibition was reduced by use of a higher concentration of PCR enzymes. The results suggest that further optimization of PCR assays for porcine oral fluids is needed and that laboratories should not assume that methods optimized for detection of virus in serum will perform equally with porcine oral fluids.

Introduction

Oral fluid is the liquid in the buccal cavity collected by use of an absorptive device.[2] The fluid is composed of saliva produced by the salivary glands and transudate that originates from the circulatory system. The diagnostic value of oral fluid reflects the presence of pathogens and/or antibodies produced locally or derived from the circulatory system. In human diagnostic medicine, both polymerase chain reaction (PCR)- and antibody-based assays have been modified and optimized for testing oral fluids for a wide variety of pathogens.[11] The low cost of oral fluid samplings in combination with the availability of assays optimized for testing oral fluid specimens has made it possible to conduct large surveys for infections of public health importance (e.g., HIV in Africa and measles in Europe).[4,5,12]

Veterinary medicine has been slow to adopt this sampling method, but testing of pen-based oral fluid samples has been shown to be effective for monitoring the circulation of *Porcine reproductive and respiratory syndrome virus* (PRRSV; order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus*) and other infectious agents in populations of pigs such as *Porcine circovirus-2*.[9,10] To date, the work has shown that, as in the case of human diseases, oral fluid sampling offers an efficient, cost-effective approach for the detection of economically important infectious diseases of pigs.[11] For porcine diagnostics, however, the process of optimizing assays for the oral fluid matrix and assessing their diagnostic performance has only been undertaken recently. Therefore, the objective of the present study was to evaluate nucleic acid extraction procedures and PCR protocols to identify the optimum combination for detection of PRRSV in oral fluid samples.

Materials and methods

Experimental design

The diagnostic performance of 5 extraction protocols and 2 reverse transcription (RT)-PCR protocols for PRRSV were evaluated using oral fluid samples from pigs of known PRRSV exposure status. Extraction protocols were selected based on the researchers' familiarity with the procedures and compatibility with high-throughput, semiautomated systems.

The oral fluid samples used in the present study were derived from PRRSV-inoculated pens collected up to 4 weeks postinoculation.[10] Pigs were randomly assigned to treatment groups and then intramuscularly inoculated on day 0 with 2 ml of a preparation containing $1 \times 10^{1.7}$ 50% tissue culture infective dose ($TCID_{50}$) of PRRSV per ml. Serum samples collected 8 days before the start of the experiment and day 0 were assayed and determined to be negative by enzyme-linked immunosorbent assay (ELISA). Serum samples collected at day 10 were ELISA positive, confirming the presence of PRRSV infection.[10] Oral fluid samples were collected as described elsewhere.[10] A subset of oral fluid samples (n = 106), including 43 samples from negative control pens and 63 samples from inoculated pens, was selected to evaluate extraction and real-time RT-PCR protocols in the present study. The 63 samples from inoculated pens ranged from sampling dates between 1 and 4 weeks postinoculation and were selected to represent a range of likely PRRSV nucleic acid concentration.

To reduce testing bias and variation, 1) oral fluid samples were completely randomized and blinded prior to testing; 2) extraction protocols were performed in

random order; 3) within each protocol, extractions on the 106 samples and extraction controls were performed start-to-finish in the same day; and 4) the 5 RNA extracts from each sample (1 for each extraction protocol) were re-arrayed to a single PCR plate to control for potential plate-to-plate variation in PCR master mix or real-time RT-PCR analysis settings between plates. Each sample was assayed 1 time by each combination of extraction and real-time RT-PCR procedure. Results were statistically analyzed to determine which procedures provided the best diagnostic performance.

Nucleic acid extraction protocols

Five different nucleic acid extraction protocols were evaluated. Protocols differed in starting sample volume, the volume of lysate used in the extraction, final elution volume, and in the method of RNA capture (Table 1).

Nucleic acid extraction protocol A1. Protocol A1 was based on a commercial kit[a] and was performed according to the manufacturer's instructions, with the exception of the preparation of the lysis/binding solution and the lysis step. For the lysis/binding solution, 40 ml of lysis/binding solution was combined with 623 µl of carrier RNA (without the addition of isopropanol), mixed thoroughly, and stored at room temperature until use. All other reagents were prepared according to the kit insert. For the lysis step, 175 µl of oral fluid sample was added to 235 µl of the lysis/binding solution in a deep-well plate.[c] Plates were covered with a seal,[g] mixed at 1000 rpm on an orbital plate shaker[d] for 5 min, and then centrifuged at 2,500 × *g* for 6 min. A volume of 115 µl of lysate was then added to each well of a new deep-well plate to which 65 µl of isopropanol[b] and 20 µl of magnetic beads mix had

already been added. The plate was loaded onto a semiautomated nucleic acid purification system[e] along with 2 plates of 150 µl of wash solution 1, 2 plates of 150 µl of wash solution 2, and a final plate of 90 µl of elution buffer, as per kit instructions. Extraction was then completed on the semiautomated system, using program "AM_1836_DW50_v2."[f] Thereafter, the elution plate was sealed using a plate sealer[g] and frozen at –80°C until assayed by real-time RT-PCR.

Nucleic acid extraction protocol A2. Protocol A2 was based on a commercial kit[a] and was performed according to the manufacturer's instructions, with the following exceptions. Lysis/binding buffer was prepared as described in protocol A1. All other reagents were prepared according to the kit insert. For the lysis step, 300 µl of sample was added to 450 µl of prepared lysis/binding solution in a deep-well plate.[c] Plates were covered with a seal[g] and mixed at 1100 rpm on an orbital plate shaker[d] for 5 min, and then centrifuged at 2,500 × *g* for 5 min. A volume of 600 µl of lysate was then added to each well of a new deep-well plate to which 350 µl of isopropanol[b] and 20 µl of bead mix had already been added. The plate was loaded onto a semiautomated nucleic acid purification system[e] along with 2 plates of 300 µl of wash solution 1 and 2 plates of 450 µl of wash solution 2, and a final plate of 90 µl of elution buffer. Extraction was then completed on the semiautomated system, using program "AM1836 DW 300 v2."[f] Thereafter, the elution plate was sealed using a plate sealer[g] and frozen at –80°C until assayed by real-time RT-PCR.

Nucleic acid extraction protocol A3. Protocol A3 was based on a commercial kit[h] and was performed according to the manufacturer's instructions using the protocol for "Disruption of Liquid Samples." All reagents were prepared according to

the kit insert. In brief, 175 µl of oral fluid sample was added to 235 µl of lysis/binding solution in a tube containing zirconia beads for sample disruption. Bead beating was carried out using a high-speed shaker[i] for 15 min followed by centrifugation to pellet the beads. A volume of 115 µl of lysate was transferred to a plate containing 65 µl of isopropanol[b] and 20 µl of magnetic bead mix. The plate was loaded onto a semiautomated nucleic acid purification system[e] along with 2 plates of 150 µl of wash solution 1, 2 plates of 150 µl of wash solution 2, and a final plate containing 90 µl of elution buffer. Extraction was then completed on the semiautomated system, using program "AM1840 DW 50 v2."[f] Thereafter, the elution plate was sealed using a plate sealer[g] and frozen at –80°C until assayed by real-time RT-PCR.

Nucleic acid extraction protocol B1. Protocol B1 was based on a commercial kit[j] and was performed according to the manufacturer's instructions using the protocol for "Cell-Free Body Fluids." In brief, 100 µl of oral fluid sample was added to 40 µl of proteinase K[j] in a deep-well plate[c] followed by addition of 600 µl of buffer, isopropanol, and magnetic bead solution (buffer RLT[j]). The plate was loaded onto a semiautomated nucleic acid purification system[e] along with 2 wash plates containing 500 µl of buffer RPE,[j] 1 plate containing buffer AW1,[j] and a final plate containing 75 µl of elution buffer,[j] per kit instructions. Extraction was then completed on the semiautomated system, using program "KF96 Vet 100."[k] Thereafter, the elution plate was sealed using a plate sealer[g] and frozen at –80°C until assayed by real-time RT-PCR.

Nucleic acid extraction protocol B2. Protocol B2 was based on a commercial kit[l] and was performed according to the manufacturer's instructions. In brief, 140 µl

of oral fluid sample was added to 560 µl of lysis buffer in a 1.5-ml centrifuge tube, mixed for 15 sec by pulse vortexing, and incubated at room temperature (25°C) for 10 min. Thereafter, 560 µl of ethanol[m] was added to the sample and mixed for 15 sec by pulse vortexing. The solution was transferred to a spin column[l] and was centrifuged at 6,000 × *g*. The process was repeated once with filtrate being discarded. Columns were then washed with 500 µl of buffer AW1,[l] centrifuged at 6,000 × *g*, and the filtrate and collection tube discarded. Five hundred microliters of AW2[l] buffer was then added to each spin column, centrifuged at 20,000 × *g*, and the filtrate and collection tube discarded. Last, a new collection tube was attached, and 60 µl of room temperature elution buffer[l] was added to each spin column. Columns were centrifuged at 6,000 × *g* for 1 min, after which collection tubes were closed and frozen at –80°C until assayed by real-time RT-PCR.

Nucleic acid extraction quality control. Quality control of the extraction process included 1 negative control (nuclease-free water) and 2 positive controls on each extraction plate or run. Positive controls consisted of a PRRSV field isolate (ISU-P) diluted in Eagle minimum essential media[n] to 2 levels: a “low positive” control ($1 \times 10^{1.0}$ $TCID_{50}$ per ml) and a “high positive” control ($1 \times 10^{2.2}$ $TCID_{50}$ per ml).

PRRSV RNA amplification and detection via real-time PCR. Real-time RT-PCR was performed with commercially available reagent sets,[o] in which North American and/or European PRRSV RNA were reverse-transcribed into complementary DNA and amplified by Taq DNA polymerase in a single-tube, one-step differential real-time RT-PCR reaction. Detection of amplified target was

accomplished by hydrolysis probe chemistry. The master mix also contained primers and probes targeting an internal positive control RNA sequence.[p] The internal control RNA[p] was spiked into the real-time RT-PCR master mix at a concentration of 100 copies per µl to monitor PCR amplification and allow for detection of failed PCR reactions. The following master mixes were evaluated.

1× master mix. The 1× master mix was prepared according to the manufacturers' instructions[f] with the following component volumes per well: 12.5 µl of 2× RT-PCR buffer, 2.5 µl of 10× PRRSV primer probe mix, 1.25 µl of 20× multiplex RT-PCR enzyme mix, 0.35 µl of a solution containing 100 copies per µl of internal control RNA, and 0.4 µl of nuclease-free water.

2× master mix. The 2× master mix was from the same manufacturer[f] but prepared with the following component volumes per well: 12.5 µl of 2× RT-PCR buffer, 2.5 µl of 10× PRRSV primer probe mix, 2.5 µl of 20× multiplex RT-PCR enzyme mix (double the amount in 1× master mix), and 0.35 µl of a solution containing 100 copies per µl of internal control RNA.

Thereafter, the following conditions were the same for both experiments: 17 µl of final master mix and 8 µl of RNA extract was placed in each well of a 96-well PCR plate.[q] Real-time RT-PCR was then performed using a 96-well real-time thermal cycler[r] using the following cycling conditions: 1 cycle at 45°C for 10 min, 1 cycle at 95°C for 10 min, 40 cycles of 97°C for 2 sec, 60°C for 40 sec. Quality control for the PCR reaction included nuclease-free water as negative amplification control in addition to a positive amplification control provided by the manufacturer.[p]

Analysis of real-time amplification curves was performed using commercial thermal cycler system software.[r] "Auto Baseline" was used to determine fluorescence baselines and a "Manual Ct" threshold was set for each run, adjusted such that the threshold was within the linear portion of the amplification curve from positive amplification controls.[p] The threshold for internal controls was set in the same fashion using amplification curves from wells that contained negative amplification controls. Samples with threshold cycles (Cts) of <40 cycles were considered positive and those with Cts ≥ 40 were considered negative.

Statistical analysis

Analysis of the real-time RT-PCR results was performed using statistical software.[s] The Cochran Q test was used to detect significant differences in the proportion of PRRSV PCR positives among the 10 protocols (5 extraction protocols × 2 master mixes) evaluated. The protocol with the highest proportion of positives in oral fluid samples from PRRSV-inoculated pens of pigs was then compared pair-wisely with the other 9 protocols using the McNemar test. Analysis of the internal control data was performed by the McNemar test for the qualitative (positive/negative) results; differences in quantitative results (Ct values) were analyzed using both the signed rank test on Ct differences between experiments and a likelihood ratio test for homogeneity of variances.

Results

Positive and negative extraction and amplification controls were valid for all extraction protocols and PCR plates. Overall, 45 out of 63 (71%) samples from pens

of PRRSV-challenged pigs tested positive on at least 1 protocol. Among oral fluid samples from negative control pens, 7 out of 430 tests (43 samples × 5 extractions × 2 RT-PCR assays) produced positive results (Table 2), for a false-positive rate of 1.6% (specificity = 98.4%).

Results and statistical comparisons for all combinations of extraction and PCR protocols are listed in Table 2. An overall assessment of the results indicated significant differences in detection level among protocols (Cochran Q statistic = 41.873, $p < 0.0001$). The combination of extraction A2 and the 2× master mix produced zero false-positive reactions in negative control oral fluid samples and the most positive reactions in oral fluids from PRRSV-inoculated pens. This protocol was significantly better ($p \leq 0.05$) than 7 of the 9 other protocol combinations when the results from the PRRSV-inoculated pens were compared in a pair-wise fashion using the McNemar test.

Using the 1× master mix in the PCR reaction, 4 of the 5 extraction methods had 2 or more negative control oral fluid samples in which the internal control was not detected (Table 3). The 2× master mix, containing twice the enzyme as the 1× master mix, eliminated failed internal controls in 3 of the affected protocols and reduced the number of failed internal controls in the fourth. The McNemar test calculated on the basis of a total of 11 and 4 failed internal control reactions in the 1× and 2× master mixes, respectively, found that the difference in proportions was statistically significant ($p = 0.0082$).

A box-and-whisker plot of Ct values for the internal control RNA in negative control oral fluids ($n = 43$) is shown in Figure 1 for every combination of extraction

and PCR protocols. The mean Ct value for the 1× master mix was 34.41 (95% confidence interval [CI]: 34.09, 34.72) and 34.79 for the 2× master mix (95% CI: 34.69, 34.89) and were not significantly different at a $p \leq 0.05$. To assess differences in dispersion of internal control Ct values, a likelihood ratio test for homogeneity of variances was performed on Ct values of internal controls and showed that the 2× master mix significantly reduced variation ($p \leq 0.05$) for every extraction protocol.

Discussion

The objective of the current study was to evaluate RNA extraction and PCR protocols for the detection of PRRSV in porcine oral fluids. Although a relatively small number of procedures were tested, the results showed marked differences among extraction protocols, PCR protocols, and combinations thereof in the oral fluid matrix. Nucleic acid extractions were conducted as recommended by the manufacturers, but sample volumes, reagent volumes, and other factors varied among protocols (Table 1). This nonuniformity precluded the possibility of a detailed analysis of all protocol variables that could potentially impact assay performance.

The use of an internal positive control in the PCR assay made it possible to determine when the reaction failed and to identify false-negative results. Each of the 5 extraction methods produced the same or fewer failed internal control reactions when the concentration of PCR enzymes in the master mix was doubled. Simultaneously, the 2× master mix resulted in a statistically significant reduction in Ct variation in the internal control reactions and the same or higher rates of positivity in samples from PRRSV-challenged pens. Cumulatively, these results suggested that the PCR reaction was partially inhibited by factors in the oral fluid matrix and

that inhibition was reduced by use of the 2× enzyme mix, but important questions remain unresolved. For example, oral fluid volumes for nucleic acid extraction protocols A1, A2, and A3 were 175 µl, 300 µl, and 175 µl, respectively, but detection of PRRSV using the 1×master mix was equivalent. In contrast, the detection level for protocol A2 greatly improved with the 2×master mix, but detection for protocols A1 and A3 did not. One possible explanation for this result is that the larger initial sample volume in protocol A2 (300 µl) eluted both more RNA and more endogenous inhibitors. Following this logic, doubling the concentration of PCR enzymes may have compensated for the greater concentration of inhibitors and allowed for improved RNA detection.

Polymerase chain reaction inhibition can manifest as complete reaction failure (false negative) or as reduced analytical sensitivity.[13] Three mechanisms of inhibition proposed include failure of lysis, nucleic acid degradation and capture, and polymerase inhibition.[13] Inhibition of polymerase activity is the most common type of PCR inhibition.[3] The presence of PCR inhibitors was previously reported in human oral fluids,[1,7,8] and feces are known to have a similar effect.[6] Because porcine oral fluid samples often contain some level of fecal matter and other environmental contaminants, inhibitors of this sample type are of particular concern. The identity and mechanism(s) of endogenous PCR inhibitors in swine oral fluid remain unknown. In human oral fluids, polysaccharides were believed to inhibit PCR; this inhibition was overcome with the use of a chelating resin.[t,8] Other approaches to overcome PCR inhibition in other specimen types have been described, including dilution of template prior to PCR, addition of bovine serum albumin during

amplification, and use of PCR enzymes designed for improved resistance to inhibition.[13] Further work is necessary to determine which of these strategies would be most effective.

Testing of porcine oral fluid samples by real-time RT-PCR has been increasingly adopted by swine producers and veterinarians as a technique for monitoring the circulation of PRRSV.[11] The growing use of oral fluid diagnostic specimens justified comparison of nucleic acid extraction and real-time RT-PCR protocols for the oral fluid matrix. The results of the current study clearly showed improvements in PRRSV PCR assay protocols for swine oral fluid, but also suggest that further assay optimization is needed.

Table 1. Comparison of nucleic acid extraction protocols.

Extraction protocol	Sample volume (µl)	Lysis solution volume (µl)	Lysate used (µl)	Elution volume (µl)	RNA capture mechanism	Semiautomated?
A1	175	235	115	90	Magnetic bead	Yes
A2	300	450	600	90	Magnetic bead	Yes
A3	175	235	115	90	Magnetic bead	Partially
B1	100	640	740	75	Magnetic bead	Yes
B2	140	1,120	1,260	60	Silica gel	No

Table 2. Comparison of extraction and polymerase chain reaction protocols on *Porcine reproductive and respiratory syndrome virus* (PRRSV) real-time reverse transcription polymerase chain reaction–positive reactions in oral fluid samples.

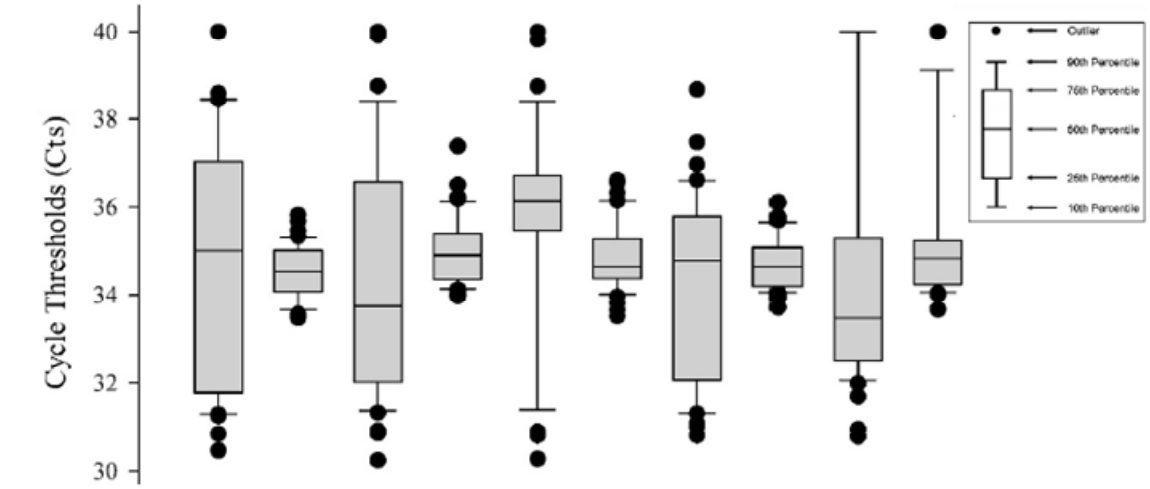
	1× master mix		2× master mix	
Extraction protocol	Negative control oral fluid samples (n = 43)	Oral fluid samples from PRRSV-inoculated pigs (n = 63)*	Negative control oral fluid samples (n = 43)	Oral fluid samples from PRRSV-inoculated pigs (n = 63)*
A1	0	27 (43)	2	27 (43)
A2	0	23 (37)†	0	35 (56)
A3	0	23 (37)†	1	24 (38)†
B1	1	11 (17)†	0	20 (32)†
B2	3	15 (24)†	0	21 (33)†

* Values in parentheses are percentages.
† Significantly different ($P \leq 0.05$) from best protocol (A2, 2× master mix) based on McNemar test.

Table 3. Count of failed internal control* reactions in negative control oral fluid samples (n = 43).

	Master mix	
Extraction protocol	1×	2×
A1	2	0
A2	2	0
A3	2	0
B1	0	0
B2	6	4
No. of samples in which internal control failed on ≥1 extraction protocol†	11	4

* Applied Biosystems, Foster City, CA.
† McNemar test (P = 0.0082) comparing 1× and 2× master mixes was calculated on the basis of a total of 11 and 4 samples, respectively, with failed internal control reactions. That is, samples with failed internal control reactions in more than 1 extraction protocol were only counted once in the analysis.



Extraction	A1	A1	A2	A2	A3	A3	B1	B1	B2	B2
Master Mix	1X	2X	1X	2X	1X	2X	1X	2X	1X	2X

Figure 1. Internal control threshold cycle values by extraction protocol.

Acknowledgements

The authors thank Amanda Manolis (Applied Biosystems, Foster City, CA) for providing extraction kits and PRRSV PCR reagents as well as Paul Pearson (Qiagen Inc., Valencia, CA) who provided extraction kits. The study was supported in part by Pork Checkoff funds distributed through the National Pork Board, PO Box 9114, Des Moines, IA 50306 and the PRRS CAP, USDA NIFA Award 2008-55620-1932. Finally, the authors thank the faculty and staff of the Iowa State University Veterinary Diagnostic Laboratory for advice and technical support.

Sources and manufacturers

a. MagMAX™ -96 Viral RNA Isolation Kit, Applied Biosystems, Foster City, CA.

b. 100% ACS grade isopropanol, Fisher Scientific Co., Pittsburgh, PA.

c. Polypropylene Microtiter® Microplates, Thermo Fisher Scientific Inc., Waltham, MA.

d. Titer plate shaker, Thermo Fisher Scientific Inc., Waltham, MA.

e. KingFisher® 96 magnetic particle processor, Thermo Fisher Scientific Inc., Waltham, MA.

f. Applied Biosystems, Foster City, CA.

g. AluminaSeal™, Diversified Biotech, Dedham, MA.

h. MagMAX™ Total Nucleic Acid Isolation Kit, Applied Biosystems, Foster City, CA.

i. TissueLyserII, Qiagen Inc., Valencia, CA.

j. BioSprint® 96 One-For-All Vet Kit, Qiagen Inc., Valencia, CA.

k. Qiagen Inc., Valencia, CA.

l. QIAamp® Viral RNA Mini Kit, Qiagen Inc., Valencia, CA.

m. 100% Ethanol ACS grade, Sigma-Aldrich, St. Louis, MO.

n. Eagle's Minimum Essential Media (MEM), Sigma-Aldrich, St. Louis, MO.

o. TaqMan® NA and EU PRRSV Reagents, Applied Biosystems, Foster City, CA.

p. TaqMan® NA and EU PRRSV and Xeno™ RNA Controls, Applied Biosystems, Foster City, CA.

q. 96-well Fast PCR plates, Applied Biosystems, Foster City, CA.

r. 7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA.

s. SAS® Version 9.2, SAS Institute Inc., Cary, NC.

t. Chelex® 100, Bio-Rad Laboratories, Hercules, CA.

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CHAPTER 3. PRRS VIRUS SEQUENCING FROM ORAL FLUIDS

A paper to be submitted to the Journal of Veterinary Diagnostic Investigation

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Abstract: Sequencing open reading frame 5 (ORF5) of the PRRS virus is a commonly used molecular diagnostic test for virus characterization and epidemiologic investigations in the United States. Although, attempts in recent years to obtain PRRS virus sequencing from swine oral fluids has been less successful than serum, limiting the cost effectiveness of using this specimen type in disease eradication efforts. It is hypothesized that polymerase-chain reaction (PCR) inhibition is one of many possible contributing factors reducing the efficiency of generating ORF5 PCR product prior to Sanger sequencing. In this study, viral RNA was extracted from 156 PRRS PCR positive field samples received by the Iowa State University Veterinary Diagnostic Lab (ISUVDL) and tested with three different modifications of the PCR reaction; “regular” or standard method, “diluted” extract prior to PCR, and “tough” or inhibition-resistant PCR enzyme mix. Success rate of generating PCR product was evaluated along with concentration of amplified product. For further confirmation, sequencing was attempted on a subset of

samples. Target amplification was successful in 94 of the 156 (60%) samples by at least one method. The 'tough' method proved to amplify from the most samples with 39% detected, followed by the 'diluted' method with 35% detected, and finally the 'regular' method with 17% detected. The "diluted method" yielded the highest cDNA concentration of the target band, resulting in a significantly higher success rate of amplifying the target than the 'regular' method, and had the highest proportion of samples successfully sequence.

Introduction

PRRS virus detection by real-time PCR and subsequent sequencing of open reading frame (ORF) 5 are commonly requested diagnostic tests. PRRS Sequencing is an integral component of eradication efforts used to evaluate new virus introduction and monitor epidemiology of virus spread.[3] In short, sequencing involves extraction of viral RNA from the sample matrix, followed by reverse transcription-polymerase chain reaction (RT-PCR) to amplify the target region of the virus, then purification of the amplified product, and submission to a core sequencing lab where Sanger sequencing is used to generate sequencing data. Data is subsequently downloaded by the diagnostic lab where it is compiled and further characterized by comparing to other strains in dendrogram or sequence homology tables.

Pen-based oral fluid samples have been shown to be effective for monitoring the circulation of PRRSV and other infectious agents, such as PCV2, in pig populations.[11,12] PCR inhibitors have previously been reported in human oral fluids

[1,8,11,17], in feces[6] and animal feed[7] which is important because swine oral fluids may contain a mix of these components due to the manner in which they are collected.[12] PCR inhibitors are of concern for their role in potential false-negative reactions or reduced analytical sensitivity.[2,13] A previous study reports an optimized RNA extraction and PCR method for PRRS detection by real-time RT-PCR but that test is separate from the sequencing reaction.[2]

While detection has been improved, subsequent virus sequencing from the oral fluid sample matrix has proven difficult. A subset of PCR-positive (Ambion® MagMAX™ pathogen RNA/DNA kit, Life Technologies™ Carlsbad, CA) oral fluid samples submitted to the ISUVDL in 2011 was tracked for sequencing success rates. Of the 1217 PRRS PCR positive oral fluid samples submitted and tested as previously described[2], 50% (609) could not be sequenced. In the same time period, 1665 sera were submitted, of which only 16% (262) were unable to be sequenced (unpublished). The relatively low success rate of sequencing from oral fluids has frustrated efforts to more widely use this specimen type in eradication and other diagnostic efforts. A number of potentially important variables may be contributing, including; lower concentration of PRRS virus in oral fluid, sample degradation during transport, and lack of optimization of the test for this new sample type. Freezing of oral fluid samples has been reported to improve sequencing success rate, though overall success rate was still limited.[15]

The goal of this study was to evaluate three RT-PCR mastermixes for their success in generating the initial ORF5 PCR amplicon commonly used for sequencing. It is assumed that improving amplification and cDNA yield in this step

will improve overall success rate of PRRSV sequencing from oral fluids. The correlation of initial screening PCR Ct value to success in ORF5 cDNA amplification across treatments was also evaluated.

Materials and methods

Experimental design

The impact of three treatments on successful RT-PCR amplification of the ORF5 target and the yield of cDNA were evaluated as indicators of likelihood of overall success of PRRS Sequencing. The method currently used by the ISUVDL ('regular') was evaluated along with two alternative RT-PCR methods ('diluted' and 'tough'). These methods were selected based on earlier reported strategies to overcome PCR inhibition including inhibition-resistant enzymes, [7,14,30] and dilution of inhibitors prior to PCR.[4,14]

Samples

The swine oral fluid samples used were selected from clinical specimens submitted to the ISUVDL in 2011 with selection criteria of having an initial real-time PRRS PCR positive test performed as previously described. Briefly, a large volume magnetic bead extraction with modified lysis buffer followed by use of a commercially available real-time PCR reagent set for screening of PRRSV RNA (Ambion® MagMAX™ pathogen RNA/DNA kit, Life Technologies™ Carlsbad, CA).[2] A total of 156 samples from 57 different client submissions were used in this study. Initial screening PRRS PCR cycle quantity (Cq) values ranged from 21 to 37 with a

mean Cq of 32. Field samples as opposed to research samples were intentionally used in this study as a means of better representing samples received by diagnostic labs in contrast to spiked samples or laboratory-challenge study samples.

Nucleic acid extraction

Total RNA was extracted from oral fluid as previously described[2] using a commercial extraction kit for oral fluid (Ambion® MagMAX™ pathogen RNA/DNA kit, Life Technologies™ Carlsbad, CA). The extraction was completed using an automated particle processor (Kingfisher-96 Magnetic Particle Processor, Thermo Scientific, Asheville, NC).[2] Samples were frozen after initial diagnostic testing and were then kept frozen at -80°Cprior to initiating the study. All samples were extracted on the same day as described below and 3 equal aliquots of RNA extract were immediately frozen at -80°C. After thawing, ORF5 RT-PCR was performed as described below.

'Regular' PCR

An aliquot of RNA extract was used as template for conventional ORF5 RT-PCR carried according to the standard protocol used by the ISUVDL during the 2011 period. This PCR reaction uses qScript Custom 1-Step RT-PCR reagents from Quanta Biosciences (Gaithersburg, MD), mixed in the following per-reaction amounts: 7.2µl of RNase-free water, 12.5µl of One step MM component, 0.4µl of 20 µM P5F2 Forward primer(5'-AAG GTG GTA TTT GGC AAT GTG TC-3') , 0.4 µl of 20 µM P5R2 Reverse primer (5'-GAG GTG ATG AAT TTC CAG GTT TCT A-3') @ , and 0.5 µl qScript One-Step RT. After mixing reagents, 21µl of this mix was

aliquoted into a 96-well reaction plate and 4µl of RNA extract was added from each sample. Thermal cycling conditions were one hold of 48°C 20 min, one hold of 94°C 3 min, 45 cycles: 94°C 30 sec, 50°C 50 sec, 68°C 50 sec, and one hold of 68°C 7 min, followed by 4°C hold. The same primer sequences, which are expected to produce a 1082 base-pair (bp) band, were used for all three PCR treatments.

'Diluted' RNA Extract PCR

Based on earlier work by Gallup[4] the "inhibitory characteristic" of the study samples was evaluated to arrive at the optimal dilution of RNA extract to use for subsequent PCR. This approach seeks the optimal intersection of inhibitor and target dilutions. Sixteen samples were randomly chosen from the 156 total samples using one sample only from a given accession. Loosely following the methodology of the "P-Q program"™ described by Gallup elsewhere[4], serial dilutions of extract were used as template for the 'regular' ORF5 PCR method described above. RNA extract was diluted in PCR-grade water in the following six dilutions: undiluted, 1:2, 1:4, 1:8, 1:16, and 1:32. DNA concentration of the resulting PCR product was measured using the QIAxcel capillary electrophoresis system (Qiagen, Valencia CA) and the consensus of 'best' dilution among all 16 samples was chosen. An example of results of the dilution series from one of the 16 samples is shown in Diagram 1 below. The dilution that had the highest DNA yield on average of the 16 samples was used hereafter. Dilution of all RNA extracts at this level (1:2 in Molecular-grade water) prior to PCR constituted the 'diluted' method with all other componentsequal to the 'regular' method described earlier.

'Tough' mastermix PRRS PCR

An alternate set of PCR reagents containing inhibition-resistant enzyme mix was used for the 'tough' treatment. Reagents were qScript XLT One-Step RT-PCR kit from Quanta Biosciences (Gaithersburg, MD). All reaction volumes were the same as the 'regular' PCR method described earlier but with the substitution "Tough-mix" RT-PCR reagents from Quanta Biosciences (Gaithersburg, MD).

Electrophoresis

Amplified DNA detection and quantification was carried out using a QIAxcel capillary electrophoresis instrument and associated ScreenGel software per manufacturer instructions.[13] The DNA Screening kit was used along with QX Alignment Marker 15bp / 3kb and a FX174 / Hae III ladder. Sample PCR product was diluted 1:5 and the ladder diluted 1:3.33 in DNAse free molecular grade water per instructions.[13] The DNA ladder had a final concentration of 30ng/µl and served both as size detection and quantification standard. Run parameters were: Method AM420, 5kV Injection and Separation Voltage, 8.5 Acquisition Rate [Hz], 0.3 sec Rise Time, 10 sec Injection Time, and 425 sec separation time. For analysis, 0.25 sec minimum separation 3.38% positive threshold and 40 sec Baseline Filter window.

After initial analysis, samples were re-analyzed such that all treatments of the same sample were examined for size and concentration together in one analysis run of the software to limit any between-run variation present in the system. QIAxcel ScreenGel™ software was used to detect, determine size, and quantify relative

concentration of products. DNA concentration reported was reported in units of as-found ng/µl and do not account for sample dilution as only relative differences among treatments were of interest.

A single band closest to the target size of 1080bp was selected when multiple bands for a given sample were observed. Bands within 20% of the target size (900-1300bp) detected during analysis were considered "positive" based on stated inaccuracy limits of the method used.[13] The assumption that a fragment in this size range would generate a successful sequence was tested on a subset of 47 samples which were selected from a range of product sizes within each treatment. These were submitted to a DNA core facility for sequencing, compiled and confirmed as PRRS virus by aligning with VR-2332 PRRS using Lasergene sequence alignment software (DNA Star, Madison, Wisconsin).

Statistical Analysis

Significance of the number of ORF5 PCR positive samples was first evaluated using Cochran's test for detecting differences among all treatments, followed by pair-wise McNemar's test for each combination. Differences in cDNA yield among treatments were first evaluated using Cochran's Mantel-Hanzel test followed by pair-wise Wilcoxon signed-rank test for each combination.

Lastly, the correlation of initial screening real-time RT-PCR Cq value to likelihood of generating the target-sized ORF5 PCR product was evaluated among the three treatments. A mixed-effect logistic regression model was created with treatment, Cq, and their interaction as fixed effects and sample number as the

random effect. This was an effort to see if any of the methods improved this correlation. Cq values are often used by submitting veterinarians to choose the sample with the most virus within an accession for sequencing, however an unpredictable success rate of sequencing has been observed with oral fluids. Statistical analyses were performed using SAS® Version 9.2 (SAS® Institute Inc., Cary, NC, USA).

Results

Optimal dilution for "Diluted" treatment

RNA extract from 16 samples were each tested using the 'regular' ORF5 RT-PCR method to find the optimal dilution which minimized impact of PCR inhibitors as described above. cDNA concentration of the band in the 900-1300bp range closest to 1080bp were evaluated and the strongest concentration chosen for each sample. In total 12 of the 16 samples successfully amplified in at least one of the dilutions; four samples did not amplify at any dilution. Results of the dilutions yielding the highest PCR product concentration were follows: two samples were best undiluted, nine samples were best diluted 1:2, one sample was best diluted 1:16, and no samples had highest concentration at the 1:4 or 1:32 dilution. A 1:2 dilution was then used as the single dilution of RNA extract for the 156 study samples in the 'diluted' treatment.

ORF5 RT-PCR Results

RT-PCR cDNA amplification of a band in the described range was successful in 94 of the 156 (60%) samples by at least one method with eight samples detected by all three methods, 33 detected by two methods and 53 detected by only one method. The 'tough' method proved to amplify the most samples with 62 detected, followed by the 'diluted' method with 55 detected, and finally the 'regular' method with 26 detected. A Cochran's Q test $p<0.0001$ result was found indicating differences among treatment groups. Pair-wise McNemar's test on number detected was performed for each treatment pair, results were as follows: regular vs. diluted $p<0.0001$, regular vs. tough $p<0.0001$, diluted vs. tough $p = 0.3778$. Pair-wise testing using McNemar's test revealed significantly improved detection for 'Tough' and 'Diluted' treatments compared to 'Regular', however no significant difference between 'Diluted' and 'Tough'. It was noted that 'Tough' generated a greater number of cDNA fragments smaller than the target size compared to 'Regular' and 'Diluted' which may have impacted its performance.

ORF5 cDNA from a subset of 47 of the 94 successfully amplified PCR products was submitted to a DNA core facility to verify that successful amplification of the ORF5 target is indicative of successful sequencing. The subset included 14 'regular', 12 'diluted' and 21 'tough' samples. Overall 23 (49%) of these samples produced a sequence that was then compared to known VR-2332 sequence and positively identified as PRRSV, and 24 did not generate useable sequence data that could be compiled. By treatment, 10 of 14 of 'Regular', 8 of 12 of 'Diluted', and 5 of 21 of 'Tough' were successful through the full sequencing process. Using these

success rates multiplied by the total number detected for each treatment, 19 (26 x 71%), 37 (55 x 67%), and 15 (62 x 24%) samples would be predicted to successfully sequence among 'regular', 'diluted', and 'tough' treatments respectively. Results of ORF5 RT-PCR success and predicted total number of successful sequences are shown in table 1 below by treatment.

The highest cDNA concentration of the single dominant band from each positive sample on average resulted from the diluted treatment with the lowest average concentration in the tough treatment. Comparison of cDNA concentration by treatment is shown in table 2 below. For cDNA concentration or yield Cochran's Mantel-Hanzel test detected significant differences among treatments. Pair-wise Wilcoxon signed-rank tests indicated significantly lower DNA yield for 'tough' treatment compared to 'regular' or 'diluted' and significantly higher concentration for 'diluted' treatment compared to 'regular' or 'tough' at $p \leq 0.05$.

Linear regression modeling demonstrated that Ct was a statistically significant predictor of generating ORF5 PCR product in 'diluted' and 'regular' but not in 'tough' treatments at $p \leq 0.05$ see table 3 below.

Discussion

A number of factors not tested in this study may account for unsuccessful sequencing from field samples. Successful amplification from field samples may have been hindered by viral RNA sequences that differed from primers used for the PCR and sequencing. The PRRS virus has the highest known mutation rate of RNA viruses[9] which often leads to failure of primers to anneal.[5] Further, primer design

has been shown to be an important aspect of a PCR assay's ability to overcome PCR inhibition.[16] In this study, a single primer set was used and follow-up with alternate ORF5 primer sets was not attempted but in practice alternate primer sets may be implemented when initial detection is unsuccessful.

The extraction method used in this study uses magnetic silica beads which have been previously shown to be effective in removal of PCR inhibitors from a number of specimen types.[14,17,27] Other modifications to this extraction platform that may also be useful but were not attempted here have been found effective with other sample types, including high-salt wash lysis buffer and additional or modified wash solutions.[27,29]

A significant improvement in amplification of target DNA was found with use of either 'tough' mastermix or dilution of extract 1:2 prior to PCR. Both inhibition-resistant polymerases[7,14,17,30] and dilution of inhibitors[4,14,17] have previously been shown to improve amplification efficiency, which is consistent with the results of this study and indicate that such strategies may improve sequencing success rates. Further work should be done to evaluate the variety of inhibition-resistant RT and DNA polymerases commercially available as only one vendor and product was evaluated in this study and there may be other alternatives that better suit swine oral fluids. [7,30] The higher number of cDNA fragments smaller than the target size suggests that this assay may have benefited from optimizing the annealing temperature to improve reaction specificity. Finally, a combination of treatments by diluting nucleic acid extract in addition to use of inhibition-resistant polymerases is worth exploring further.

Using real-time PRRS RT-PCR Cq values as a predictor of successful sequencing is common practice with serum samples. In the presence of inhibitors, however, Ct values are no longer reliable indicators of virus quantity.[4] Of the three treatments evaluated, 'diluted' produced the lowest p-value of $p = 0.0002$ in the logistic regression modeled. This supports findings of Gallup who proposed the 'CRUD rule' as "**C**q values are only **R**eliable in **U**ninhibited qPCR reactions where samples have undergone premeditatedly precise **D**ilutions."[4]

Dilution of RNA extract is fairly simple to implement and resulted in a significantly better success in amplifying the target compared to the "tough" treatment. Though not significant, dilution of the template yielded slightly higher DNA concentration than the 'tough' enzyme evaluated, and had a higher proportion of samples successfully sequenced. The dilution rate used in this study was the consensus of only a subset of 16 of the 156 samples within the study, each diagnostic specimen or source can be assumed to ideally require a different dilution.[4] For diagnostic labs, optimizing with a larger sample set may be worthwhile before selecting a standard dilution.

The assumption that poor success of sequencing PRRS virus from oral fluid was mainly a function of failed ORF5 PCR was somewhat refuted by the relatively low rate of generating a final compilable sequence. This suggests that other factors besides inhibition, such as virus degradation or inhibition of other aspects of Sanger sequencing may be at play. It is also possible that the range for "positive" band size was too wide, though it was selected based on stated inaccuracy limits of the instrument used.[13]

In conclusion, improvement of success rate in PRRS Sequencing was found possible through dilution of RNA extract prior to RT-PCR but more work is needed in order to achieve the level of success achievable from serum samples.

Figure 1 – Example of selection of “Best Dilution” of RNA extract to maximize PCR DNA yield

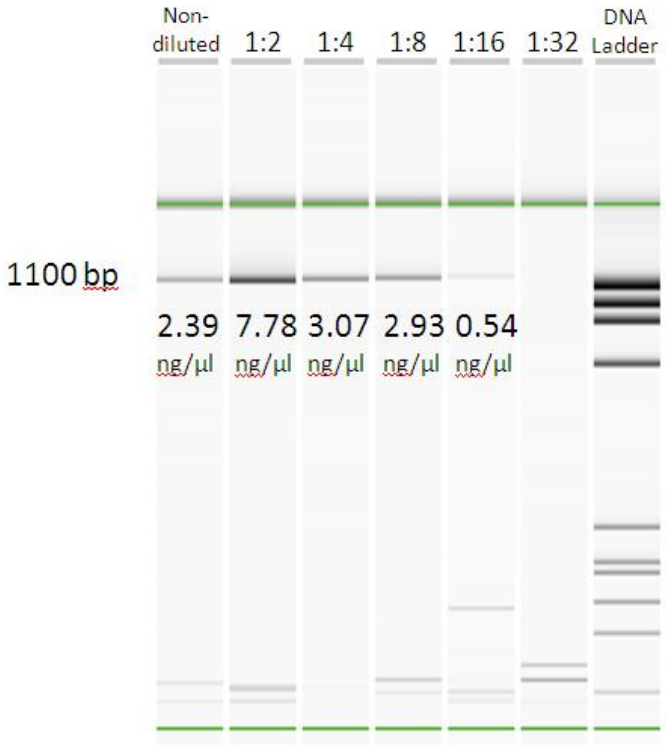

Table 1 – Percent of oral fluids 'positive' by ORF5 RT-PCR and estimated sequencing success rate

Treatment	ORF5 DNA Fragment Detected (900-1300bp)	Estimated Sequencing Success Rate*
'Regular' Mastermix	16.67% [A] (26/156)	12.2%
'Diluted' Extract 1:2 with Regular Mastermix	35.26% [B] (55/156)	23.7%
'Tough' Mastermix	39.74% [B] (62/156)	9.6%

Cochran's Q test detected differences of ORF5 DNA detected among all treatments p<0.0001

AB frequencies significantly different by McNemar's test at p<0.0001

*Subset of each treatment attempted for full sequencing. Estimated success rate = ((number sequence success / number attempted) x number ORF5 DNA detected) / 156

Table 2: cDNA concentration by treatment of single band per sample

Treatment	Mean cDNA Concentration[1] µg/µl
'Regular' Mastermix	0.773[A]
'Diluted' Extract 1:2 with Regular Mastermix	1.767[B]
'Tough' Mastermix	0.959[C]

Concentration means include zero values.

Significant difference detected among all treatments by Friedman test at P<0.0001

[ABC] Differences significant by pair-wise comparison among treatments by Wilcoxon Signed-Rank Test at p<0.05

Table 3: Cq as predictor of successful ORF5 RT-PCR by treatment

Treatment	P-value: PRRS PCR Cq as Predictor of ORF5
'Regular' Mastermix	0.0055
'Diluted' Extract 1:2 with Regular Mastermix	0.0002
'Tough' Mastermix	0.1377

*A mixed-effect logistic regression model was created with treatment, Ct, and their interaction as fixed effects and sample number as the random effect.

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CHAPTER 4. GENERAL CONCLUSIONS

General Conclusions and Future Directions

Diagnosis of PRRS virus by the latest molecular test methods may pose the biggest challenge to swine diagnostic laboratories. The work reported here lead to modest improvements in sensitivity and success rates of real-time RT-PCR and virus sequencing respectively. It is now clear that PCR inhibition is an important obstacle with this sample matrix.

PRRS viral RNA in swine oral fluids faces serious threat of degradation by consumption from contaminant bacteria, salivary enzymes, pH increases associated with bacterial growth, and likely large concentrations of nucleases capable of destroying RNA once outside the nucleocapsid. Although some work has been reported in this area, repeating this work is justified based on relatively low virus load in oral fluid alone.

Finally, although this work focused on laboratory methods to overcome ‘dirty’ field samples; cleaner on-farm collection of swine oral fluids has potential to improve the final diagnostic result. Any progress on the methodology to collect a purer sample makes efforts of lab methods more effective and should be pursued.