A Literature Review & Recommendations

The sensitivity and specificity of the collection & laboratory analysis of non-conventional Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) diagnostic samples

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EXECUTIVE SUMMARY

Canadian swine producers spend substantial time, energy and money testing for PRRS. Yet clearly there is a limit to the resources that can, and should, be devoted to PRRS monitoring. Protocols should optimize the time to detection, the probability of detection, or limit the effects of incursion per dollar spent. This can be achieved through timely collection of representative samples that are inexpensive to obtain and test. Samples that are obtainable by people with little training, or that represent many pigs, or that are stable and can be tested on an as-needed basis could all improve monitoring efficiency. The cost-effectiveness of a monitoring program could also be improved by pen-side tests, tests detecting multiple diseases, or tests that answer multiple questions about PRRS transmission and stability. This review was undertaken to describe such alternative diagnostic tests and or specimens and to evaluate what, if any, research is required before these alternatives can be recommended for PRRSV monitoring in Canada.

Specimens and tests meeting all of these criteria are described in this report. Some are ready for use in farm-level PRRS decision. But for ‘community’ PRRS decisions, such as ARC&E projects or certification projects, three actions are necessary to ensure timely and appropriate integration of these alternatives. First, producers and veterinarians must advocate for access to these tests. Secondly, they must provide the data and demand the analysis necessary to adjust for uncertainty, sensitivity and specificity. Finally, they must communicate the appropriate diagnostic outcome, which is the degree of confidence in the herd PRRS status. Without these actions, these alternative data sources risk being ignored at best and may provide misleading results at worst. Yet with such efforts, non-traditional information can substantially improve the cost-effectiveness and palatability of PRRSV monitoring.

This report describes several combinations of specimens and diagnostic tests that are not commonly used for PRRS testing in Canada. A full list is provided (Table 1) with only the most pertinent mentioned here. A pen-side immunochromatographic strip test (ICST) targeting PRRSV-specific antibodies appears close to being market ready. When approved by federal licensing agencies, this test should be closely evaluated as it may prove useful for time sensitive decisions on moving animals into stable or negative herds. Three diagnostic specimen / test combinations were identified with near-immediate potential for PRRS monitoring in Canada. These are ELISA testing on pooled serum, PCR testing on oral fluids, and ELISA testing on oral fluids. In Canada, few veterinary diagnostic laboratories offer PRRS ELISA testing on pooled serum despite strong evidence to support this approach. Laboratories may require assistance to optimize testing protocols and SP cut-offs. Most Canadian veterinary diagnostic laboratories are offering PCR testing on oral fluids but to our knowledge none are offering ELISA testing on oral fluids. Results for oral fluids testing using extraction and RT-PCR enzyme mix protocols optimized for serum vary substantially. Despite this, there have been no reports of the reliability, repeatability or reproducibility of PRRSV PCR testing on oral fluids in Canada. Hence, we recommend support for laboratories to optimize, validate, and standardize their testing and report their findings. For ELISA tests on oral fluids we recommend the literature be watched closely for studies that validate modifications to commercial tests. We believe such information is imminent. At that time, support similar to that recommended for PRRS PCR testing on oral fluids would be appropriate.
As PRRS control moves from a private-enterprise activity to a community endeavor, the industry will demand cost-effective monitoring options. With those demands comes the responsibility to ensure that laboratories and regional projects have the tools to adequately evaluate and validate testing options. These responsibilities are outlined in our 5 key recommendations.

**Recommendations**

I. Initiate a ring-test study among Canadian veterinary diagnostic laboratories to compare PRRSV results using traditional tests and specimens.

II. Support Canadian studies to validate new diagnostic tests or validate use of existing tests on novel specimens with requirements that recognized study approaches and standardized reporting be followed.

III. Ensure access to samples for validation studies and on-going proficiency panel testing for both traditional test / specimen combinations and novel alternatives.

IV. For regional control projects or community-based decisions, responsibly accept all forms of information on PRRSV status by adjusting confidence using sensitivity, specificity and uncertainty estimates obtained through ongoing validation on a representative portion of samples against a reference test and specimen.

V. Mediate discussions on the need for a PRRS diagnostic reference laboratory.
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Table 1. List of the reviewed specimens, collection techniques and tools, storage devices, and diagnostic tests reviewed for pooled and individual samples in pigs of various age groups.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Collection tool or storage device</th>
<th>Test Type</th>
<th>Animal Class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RT-PCR</td>
<td>RT-LAMP</td>
</tr>
<tr>
<td>DEFAULT = BLOOD</td>
<td>Venipuncture</td>
<td>P</td>
<td>I</td>
</tr>
<tr>
<td>Blood</td>
<td>Swabs</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Capillary tubes</td>
<td></td>
<td>I</td>
<td>N</td>
</tr>
<tr>
<td>Filter discs</td>
<td></td>
<td>I</td>
<td>N</td>
</tr>
<tr>
<td>Oral Fluid</td>
<td>Rope</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Filter disc</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Clinical specimens</td>
<td>PM Samples</td>
<td>P</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Filter discs</td>
<td>I</td>
<td>N</td>
</tr>
<tr>
<td>Abortus</td>
<td>Thoracic fluid</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td>I</td>
<td>N</td>
</tr>
<tr>
<td>Meat juice</td>
<td></td>
<td>I</td>
<td>N</td>
</tr>
<tr>
<td>Tonsil</td>
<td>Swabs</td>
<td>I</td>
<td>N</td>
</tr>
</tbody>
</table>

**P** = pooling: published evidence describing test/specimen combination in pig classes marked 'Y' using pooled samples;

**I** = individual: published evidence describing test/specimen combination in pig classes marked 'Y' but no evidence describing testing pooled samples so default is individual specimen testing

**N** = no: evidence describing the test/specimen combination

**Y** = yes: published evidence to support use of test/specimen combination in this pig class

RT-PCR: reverse transcriptase polymerase chain reaction

RT-LAMP: Reverse transcriptase loop mediated isothermal amplification

ELISA: Enzyme-linked immunosorbent assay

ICST: Immunochromatographic strip test
Table 2. List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% CI</td>
<td>95% Confidence Interval</td>
</tr>
<tr>
<td>ARC&amp;E</td>
<td>Area Regional Control and Elimination</td>
</tr>
<tr>
<td>C-US-PRRS</td>
<td>Classical Type 2 PRRS Virus</td>
</tr>
<tr>
<td>cDNA</td>
<td>Compliment Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CFIA</td>
<td>Canadian Food Inspection Agency</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dpi</td>
<td>Days Post Infection</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbtent Assay</td>
</tr>
<tr>
<td>ELISA OD</td>
<td>Enzyme-Linked Immunosorbtent Assay Optical Density</td>
</tr>
<tr>
<td>ELISA SP Ratio</td>
<td>Enzyme-Linked Immunosorbtent Assay Sample To Positive Ratio</td>
</tr>
<tr>
<td>FAD</td>
<td>Foreign Animal Disease</td>
</tr>
<tr>
<td>gel-EIA</td>
<td>Gel Enzyme Immunoassay</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>H-US-PRRS</td>
<td>Highly Pathogenic Type 2 PRRSV (nsp2 deleted)</td>
</tr>
<tr>
<td>ICST</td>
<td>Immunochromatographic Strip Test</td>
</tr>
<tr>
<td>IFA</td>
<td>Indirect Immunofluorescent Assay</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>MLV</td>
<td>Modified Live Virus Vaccine</td>
</tr>
<tr>
<td>nsp</td>
<td>Non-Structural Protein</td>
</tr>
<tr>
<td>OIE</td>
<td>World Organization For Animal Health</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pp</td>
<td>Polypeptide</td>
</tr>
<tr>
<td>PRRSV</td>
<td>Porcine Reproductive and Respiratory Syndrome Virus</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real Time or Quantitative Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RT-LAMP</td>
<td>Reverse Transcriptase Loop Mediated Isothermal Amplification</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific Pathogen Free</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median Tissue Culture Infective Dose: the amount of an virus required to infect 50% of the inoculated tissue culture cells</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>VDL</td>
<td>Veterinary Diagnostic Laboratory</td>
</tr>
<tr>
<td>VI</td>
<td>Virus Isolation</td>
</tr>
</tbody>
</table>
INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a viral disease of pigs caused by the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). The clinical signs of this syndrome include reproductive failure in breeding animals and respiratory disease in growing pigs. The clinical manifestation ranges from unapparent infection to devastating mortality. Mirroring the variation in clinical expression, infected farms report variable financial losses. Across Canada PRRS has been estimated to cause annual losses of 130 million dollars per year (Mussell et al. 2011).

The PRRS was first detected in North America in 1987 and in Europe in 1990. It has since disseminated to most swine producing countries. The PRRSV is a single stranded RNA virus classified within the Arteriviridae family. There are two distinct antigenic types (genotypes) which share only ~55 to 60% sequence homology. Initially Type 1 was found only in Europe and Type 2 in North America leading to each genotype being commonly referred to by location. This system may become confusing given that both Type 1 and Type 2 strains are beginning to be identified in Europe, North America, and Asia (Fetzer et al. 2006; World Organization for Animal Health (OEI). 2010b). To the best of our knowledge, only Type 2 PRRSV has been found circulating in pigs indigenous to Canada.

PRRSV has been referred to as a quasi-species. Genetic diversity arises through mutations and recombination (World Organization for Animal Health (OEI). 2010a). A herd and even an individual pig can be concurrently infected with multiple PRRSV strains that can include both genotypes (Oleksiewicz et al. 1998). Ongoing genetic evolution creates challenges for diagnostic test design and performance. Tests must be validated over a wide range of viruses, must undergo continual evaluation to ensure they remain current, and unexpected results must always be investigated.

The sensitivity of diagnostic tests changes over the course of infection: For direct tests, PRRSV is first detectable in serum and oral fluids within one day post infection (dpi) and peaks between 5-7 dpi (Shin et al. 2002; Wills et al. 2003). Virus concentration declines over approximately 1 month; ceasing to be predictably detected 3 to 4 weeks post infection. Most pigs will clear the virus within 3 to 4 months but persistent and sub clinical infection can last over 200 days (Batista et al. 2002; Horter et al. 2002; Lopez et al. 2004; Wills et al. 2003). Negative direct test results require concurrent antibody-based test results to clarify interpretation when the between-test interval is greater than 3 to 4 weeks,

The sensitivity of indirect or antibody based tests is also time dependent. A humoral immune response can be detected within one-week of infection and typically lasts more than 150 days (Batista et al. 2002; Horter et al. 2002; Lopez et al. 2004). Although PRRSV induces a humoral and cell mediated immune response, a protective humoral response is delayed and incomplete. The neutralizing antibody response does not occur until 21 dpi and declines more rapidly than the total antibody response. Pigs can remain persistently infected in the face of serum neutralizing antibodies (Kim et al. 2007). Additionally, while amnestic immunity is strong against homologous strains, it is incomplete against heterologous strains (Murtaugh et al. 2002). Diagnostic tests that
identify PRRSV exposure of herds based on antibody are in widespread commercial use but tests to predict cross-protective immunity are remain at the research stage.

Diagnostic tests for PRRSV have many applications including; diagnosis of clinical disease, diagnosis of production limiting disease, regional prevalence studies, regional control planning and surveillance. This review considers diagnostic tests or specimens for all of these applications except the diagnosis of clinical disease. Diagnostic test outcomes are considered for their implication in premises or herd level decisions regardless of whether specimens are collected from individuals or groups, or tests are run on specimens individually or as pools.

This review considers diagnostic test advancements that help to identify swine premises infected with or exposed to PRRSV. It also summarizes and evaluates the literature on alternatives to current mainstream diagnostic specimens for PRRSV monitoring. Definitions required to understand diagnostic test evaluation are reviewed as is the PRRSV structural biology necessary to understand differences between diagnostic tests. The industry-readiness of the described alternatives is considered and recommendations on the use of these alternatives and/or further research requirements are made.

METHODS

The inclusion and exclusion criteria were established to meet the requested scope in the call for proposals and are denoted below in Table 3. This report considered diagnostic tests that to our knowledge are not commonly available or used in Canada and are not commercial test kits. Commercially available tests are described when information on their characteristics as reference test were relevant. Given the focus on health monitoring, diagnostic tests on post-mortem tissues and specimens from clinical cases were largely excluded. Semen has historically been used as a diagnostic specimen for monitoring PRRSV freedom in boar studs. This approach has largely been abandoned in favor of serum testing based on the more rapid time to detection and higher sensitivity (Reicks et al. 2006). Given this industry experience, semen was not reviewed as a specimen.

The inclusion and exclusion criteria were adapted to search strings using Boolean operators (Table 4). Search strings were systematically applied to three databases: Agricola, EMBASE, and Med Line. Searches were restricted to publication dates between Jan.1, 2000 and present and to English language publications. Search strings were also applied ad hoc to the American Association of Swine Veterinarians (AASV) library. This library includes citations from several swine conferences that are not listed elsewhere but does not accept complex search strategies. The most recent conference proceedings of the International PRRS Symposium and Conference of Research Workers in Animal Diseases (CRWAD) were searched manually. The titles and abstracts of retrieved citations were screened manually. Those identified as potentially relevant were exported into citation management software (RefWorks© 2009) and the full citation retrieved.

In addition to the literature search, information on unpublished studies, tools, and techniques were sought by the following approaches. A call for information was distributed through list-serves offered by the Canadian Swine Health Board, American Association of Swine Veterinarians, and American Association of Veterinary Laboratory Diagnosticians. Each response was followed
up on individually. The following veterinary diagnostic laboratories and diagnosticians were contacted directly with questions regarding the current scope of offered tests and specimens for PRRSV diagnostics: Dr. André Broes, Biovet, Dr. Susy Carman, Animal Health Laboratory, Dr. Tomy Joseph, Veterinary Diagnostic Services, Manitoba Agriculture Food and Rural Initiatives, Drs. Msangu Ngleka and Dale L. Godson, Prairie Diagnostic Services. Other professional colleagues were contacted to provide experience or knowledge of specific techniques. The AASV trade show was attended and all companies offering diagnostic specimen collection products were contacted. The customer service or technical service department of commercially available diagnostic test and specimen collection tools were contacted when questions arose that were not addressed in the literature.

The literature on diagnostic tests and specimens was described. After reviewing the published literature it was evident that no diagnostic test or specimens has had sufficient research to initiate efforts for systematic review or meta-analysis. A range of the reported estimates was tabulated but as the values were not directly comparable they do not provide a consensus estimate of sensitivity or specificity.

A survey was developed to evaluate the practicality of the alternative diagnostic specimen collection tools. Four veterinary practitioners were approached to participate in a demonstration of the tools and techniques to collect specimens. The veterinary practitioners were selected to be regionally dispersed but selection was purposive. Each veterinary practitioner was asked to apply eight tools or techniques to pigs in two client herds. In each herd, the veterinarian was to work in conjunction with the staff member whom normally collects or assists with collection of diagnostics samples. Both the veterinarian and producer were requested to complete surveys (Appendix 1). Surveys were distributed for a period of four weeks. Surveys were returned electronically. Results will be summarized and described using commercially available software.
Table 3. List of inclusion and exclusion criteria used to develop Boolean search terms for literature retrieval.

<table>
<thead>
<tr>
<th>Category</th>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
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<tbody>
<tr>
<td>Pig type</td>
<td>Live domestic pigs</td>
<td>Dead domestic pigs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Feral pigs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-porcine</td>
</tr>
<tr>
<td>Specimen Type</td>
<td>Tissues, fluids, secretions</td>
<td>Environmental samples and fomites</td>
</tr>
<tr>
<td>Test Type</td>
<td>Screening and monitoring tests</td>
<td>Epidemiological tracing tests</td>
</tr>
<tr>
<td></td>
<td>Laboratory or pen-side based</td>
<td>Virus Isolation</td>
</tr>
<tr>
<td></td>
<td>PCR, LAMP, Antigen capture</td>
<td>RFLP</td>
</tr>
<tr>
<td></td>
<td>ELISA, Antibody ELISA, ICST</td>
<td>Sequencing</td>
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<tr>
<td>Study designs</td>
<td>In vitro assessments</td>
<td>Case reports</td>
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<td></td>
<td>Company marketing material</td>
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</tr>
<tr>
<td></td>
<td>Personal communication</td>
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Table 4. Boolean search strings applied to citation databases.

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<thead>
<tr>
<th>Location</th>
<th>String</th>
</tr>
</thead>
<tbody>
<tr>
<td>Key word</td>
<td>Pig* OR Pork OR Swine OR Porcine OR Sow OR Boar AND</td>
</tr>
<tr>
<td>Abstract</td>
<td>Diag* OR Specimen OR Test OR Sample OR Ig* OR antibody OR immunoglobulin virus AND</td>
</tr>
<tr>
<td>Abstract</td>
<td>Blood OR Serum OR Oral OR Fluid OR Juice OR Meat OR Swab OR venipuncture AND</td>
</tr>
<tr>
<td>Abstract</td>
<td>Sensitiv* OR Specific* OR Predictive value OR Accura* OR Precis* OR Reliab* AND</td>
</tr>
<tr>
<td>Abstract</td>
<td>PCR OR Pen-side OR ELISA OR IFA OR Chromatic AND</td>
</tr>
<tr>
<td>Abstract</td>
<td>PRRS* OR Porcine Reproductive Respiratory Syndrome virus</td>
</tr>
</tbody>
</table>
BACKGROUND

DEFINITIONS

Diagnostic tests are evaluated using terms in a very specific way. Many of these terms are used more loosely in common language and some are used differently by different specialties – namely epidemiologists and diagnosticians. The definitions, as used in this document, are described below.

The terms sample and specimen are often used interchangeably. This document defines “specimen” as a small biological sample or part taken to show the nature of the whole animal while “sample” is defined as a collection of individual units taken on the assumption that they represent the characteristics of the population (Blood et al. 1988). The method of collecting specimens will be termed a “technique” while the device or method used to contain and store a specimen between collection and testing will be defined as a “tool”. The terms “assay”, “test method” and “test” are all considered synonymous and used interchangeably (World Organization for Animal Health (OEI). 2010a). Tests that detect PRRSV particles, including nucleic acids, structural or non-structural proteins, enzymes etc. are termed “direct tests” while those that detect antibodies or a cell-mediated immune response are termed “indirect tests”.

Validity is the extent that a test measures what it is supposed to. A valid diagnostic test describes the health situation as it truly is. This is not reported as a formal measurement because in most circumstances the true health status is unknown. Accuracy is the laboratory component of validity; it is the probability that the test result reflects the true biological status of the specimen and is calculated as the sum of the properly identified true positives and true negatives divided by all samples tested (Greiner et al. 2000).

Reliability is the consistency in the measuring process. A reliable test will produce consistent results when repeated over time. This is a quantifiable characteristic. Tests can be reliable but not accurate, but cannot be accurate without being reliable. A reliable test is precise, repeatable and reproducible. Precise diagnostic tests produce a consistent result to a specific measureable degree. Repeatability is the consistency in measurement between and within runs. Reproducible is the consistency in measurement between laboratories. This review considers many studies on diagnostic test evaluation; these almost always investigate precision, often measure repeatability, but rarely investigate reproducibility. This has implications for evaluating accuracy, validity and ultimately for making industry recommendations for test use.

Diagnostic test evaluation is often conducted on experimentally spiked samples or infected animals. This eliminates uncertainty in the true status and is the simplest method to estimate sensitivity and specificity. A test must be reliable under these conditions to be considered for application in a field setting. However, it should ultimately be evaluated in the field. The results from experimentally spiked samples represent a best-case scenario because biological variation and confounders have been controlled for.

Analytical sensitivity is the lowest concentration of the test substance that can be detected. Analytical specificity is degree to which the test cross-reacts with other known pathogens. Analytical sensitivity and specificity are a narrow component of diagnostic sensitivity and
specificity. Diagnostic sensitivity (referred to in this document as sensitivity) is the conditional probability of a positive test given an animal is truly diseased. Diagnostic specificity (referred to as specificity) is the conditional probability of a negative test given an animal is truly not diseased.

Estimates of sensitivity, specificity, and validity are population specific. They are often discussed as discrete values but are actually distributed across populations. Different sensitivity and specificity estimates can be obtained for the same test due to biological differences, biased sample design, and random error (O’Connor A.M. et al. 2007). Estimates should always be reported with a description of the reference population and confidence intervals for correct application. It is also ideal to stratify these estimates across known predictors such as age, sex, and breed (Jackson et al. 2007). Bias in sensitivity and specificity are emphasized when estimating herd sensitivity and herd specificity. Bias is a systematic (rather than random) deviation from the true estimate. This is relevant to this report because the focus is on herd, or regional, inferences. The statistical methods that can adjust for this are beyond the scope of this review, but readers should be aware that estimates cannot always be applied at face value.

### PRRSV STRUCTURAL BIOLOGY

The PRRSV is a member of the *Arteriviridae* family. This family includes lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV) and simian haemorrhagic fever virus (SHFV). The *Arteriviridae* family are enveloped, positive-sense RNA viruses. The PRRSV genome is comprised of the following 9 overlapping open reading frames (ORF): 1a, 1b, 2a, 2b, and 3 to 7 (Dokland. 2010). Together ORF 1a and 1b comprise more than 75% of the genome. ORF1a encodes the polyprotein pp1a. The pp1a is presumed to be cleaved into 9 non-structural proteins which are proteolytic (nsp 1α, nsp1β, nsp 2 –nsp8). The genome encoded by ORF1b, polyprotein pp1ab, generates the non-structural proteins nsp9 through nsp12. These are involved in virus transcription and replication (Brown et al. 2009; Dokland. 2010; Music et al. 2010). ORF2 encodes for the unglycosylated E protein and the glycosylated membrane proteins GP2a, ORF3 to 5 encode for the glycosylated membrane proteins GP2a, ORF3 to 5 encode for the glycosylated membrane proteins GP3 to GP5, ORF6 encodes for the M protein which is the most conserved structural protein of PRRSV, and ORF7 encodes for the nucleocapsid (N) protein.

Genomic based tests designed to detect exposure to a wide range of PRRSV are based on highly conserved genomic segments while tests designed to distinguish between viruses are based on variable segments. The most common targets for direct PRRS detection are OFR5, ORF6, and ORF7 (Kim et al. 2007). The ORF5 encoded GP5 protein is the most variable protein of PRRSV. The ORF6 encoded M protein is the most highly conserved structural protein of PRRSV. It is not as abundantly expressed or immunogenic as the N protein. The ORF7 encoded N protein is the most abundant viral protein expressed in infected cells and also is the most immunogenic viral protein (although the antibodies are not neutralizing or protective). The N protein contains 123 – 128 amino acids and the area encoding residues 1 to 57 possess the greatest sequence differences between PRRSV strains. Hence, PCR tests targeting these sequences can be designed to detect infection with, and distinguish between, PRRSV genotypes.(Dokland. 2010; Guarino et al. 1999; Oleksiewicz et al. 1998).
For indirect testing, antibodies against the N protein are a common target to diagnose exposure to PRRSV. Tests attempting to infer information about the protective immunity against PRRSV often target antibodies against the GP5 protein (encoded by ORF5) as these are neutralizing. They arise roughly 21 dpi and last for up to 5 months (Kim et al. 2007; Loemba et al. 1996; Lopez et al. 2004). Substantial pig to pig and virus to virus variation exists in the amount and timing of antibody expression so serum neutralizing test results can be difficult to interpret on an animal level (Kim et al. 2007). The ORF4 and ORF5 are highly mutagenic; there is sufficient variation in these regions to detect different antibody responses within a genotype so these regions tend to be studied when the purpose is distinguishing between viruses or attempting to predict cross-protection (Oleksiewicz et al. 2005).

**DIAGNOSTIC TESTS**

**PRRSV DIRECT TESTS**

**VIRUS ISOLATION**

Virus isolation (VI) is rarely used for routine PRRSV diagnostics because it requires infectious virus, takes 7 to 21 days to provide results, and is labor intensive. Culture requires porcine alveolar macrophages harvested from specific pathogen free (SPF) pigs less than six to eight weeks of age. Laboratories may not have access to these pigs or cells and the susceptibility of macrophages to infection can be variable (World Organization for Animal Health (OEI). 2010b). Maintaining cell lines is an ongoing issue for laboratories as established cell lines loose susceptibility over time (Chen et al. 2008). Virus isolation is also challenging and somewhat unpredictable. Compared to RT-PCR, virus isolation has relatively low analytical sensitivity (100 fold lower than qRT-PCR) (Kleiboeker et al. 2005). It can produce biased results because certain viral strains, namely modified live vaccine strains, grow more readily than others (Kleiboeker et al. 2005). This background information on VI is presented because it is still occasionally reported as a reference test. It is not considered further in this review as an alternative test.

**VIRUS SEQUENCING & RESTRICTED FRAGMENT LENGTH POLYMORPHISM**

Genomic variation is used to understand virus transmission and to identify similarities in virus over time and space. Both virus sequencing and restriction fragment length polymorphism (RFLP) are used. Virus sequencing compares amplified nucleic acids and their corresponding amino acids in a target region based on the percent homology and clustering. The ORF5 is most commonly used (Murtaugh et al. 2001). For RFLP, enzymes are used to cut the nucleic acids and the patterns are compared. Virus sequencing is more precise than RFLP. Similar strains can have different RFLP patters and strains with a similar RFLP pattern can have sufficiently dissimilar nucleic acids to be placed in different phylogenic groupings (Larochelle et al. 2003). For these reasons, virus sequencing is becoming the recommended approach (Carman et al. 2011). Neither tool is used to monitor for the presence of PRRSV and so are not considered further in this review. They are mentioned because they have been considered a gold-standard test to confirm RT-PCR specificity (Kleiboeker et al. 2005; Oleksiewicz et al. 1998).
The reverse transcriptase polymerase chain reaction (RT-PCR) test is rapid and has high analytical sensitivity (Batista. 2005; Christopher-Hennings et al. 2002). The analytical sensitivity is further improved in nested RT-PCR and real-time or quantitative RT-PCR (qRT-PCR) reactions. The qRT-PCR can be fully automated: When applied as a single-step to detect and quantify the opportunity for cross-contamination (i.e. false positives) is limited (Lurchachaiwong et al. 2008).

The results of RT-PCR and nested RT-PCR are traditionally reported by gel-electrophoresis. In qRT-PCR viral copies are quantified by the amount of fluorescence that occurs as each cycle is completed. This can improve sensitivity because samples near the analytical threshold are identifiable and can be more closely examined to determine their true status (Kleiboeker et al. 2005). Clinically, it is unclear what viral load signifies but the quantitative outcome is useful for studies comparing viruses or studying pathogenesis, treatment and vaccine efficacy (Christopher-Hennings et al. 2004; Kleiboeker et al. 2005).

Diagnostic laboratories offering RT-PCR PRRSV tests should validate their procedures and regularly perform quality control checks. Proper validation is an intensive process requiring unbiased samples from a well described reference population that reflects the target population (Greiner et al. 2000; World Organization for Animal Health (OEI). 2010a). To achieve a precision of +/-1% for a test with an *a priori* specificity estimate of 95% requires >1800 samples while a precision of +/-5% for a test with an *a priori* sensitivity of 90% requires >100 samples (Greiner et al. 2000). It is worth noting that although test validation studies commonly confirm that a test does not cross-react with samples confirmed positive for other common swine pathogens this is not equivalent to ensuring the test does not produce a false positive on samples negative for the pathogen of interest from the target population.

The RNA extraction and RT-PCR enzyme mix protocols used affect diagnostic performance. To overcome the rigorous requirements of test validation, laboratories often use commercial kits or procedures that have been evaluated in peer-reviewed literature. The validated procedure can then be run in parallel with the currently offered test to compare performance (Carman et al. 2010). This approach alleviates some validation requirements and addresses concerns inherent with using published estimates at face value. First, diagnostic test sensitivity and specificity are population specific estimates, so laboratories must ensure that samples used in validation are representative of samples originating from their clients’ herds. Secondly, published protocols are commonly modified and all modifications must be validated. A situation commonly faced by Canadian laboratories is the need to modify fully automated procedures to perform some or all steps manually. A published example of changing the RNA extraction methods from a reported approach to a commercial kit resulted in a high rate of false positive PCR tests; the number of field tests with false positive results reported is unknown but speculated to be substantial with worldwide occurrence (Fetzer et al. 2006; Oleksiewicz et al. 1998). This emphasizes the need to be diligent in validating all protocols.

There are no formal requirements that diagnostic methods, validation procedures, or quality control testing results be made publically available; although this information should be available to those using the results (Christopher-Hennings et al. 1998). Canadian labs have the option to participate in proficiency panels for direct and indirect PRRSV testing that are offered by
American Institutions (Personal Communication; Dr. Sabrina Swenson, APHIS USDA, Diagnostic Virology Laboratory, National Veterinary Services Laboratories; Sarah Layton, Boehringer Ingelheim Vetmedica Inc.). These results are blind for participants and are not public. No Canadian studies have published a comparison of PRRSV test results between laboratories. This may be a useful exercise given the experience in Germany where a ring test found that 20% of samples sent to 13 laboratories were improperly diagnosed as PRRSV positive or negative with even lower accuracy when differentiating between Type 1 and 2 (Truyen et al. 2006). As a veterinary community, it is our responsibility to diligently question diagnostic test results and to request transparency from diagnostic laboratories regarding protocols, quality control and validation.

The analytical sensitivity of RT-PCR PRRSV tests exceeds any other diagnostic test available. An infected pig can have 1000 TCID<sub>50</sub>ml<sup>-1</sup> of viral particles in its serum while qRT-PCR can consistently detect 0.01 TCID<sub>50</sub>ml<sup>-1</sup>. So while it is possible to fail to detect an infected pig due to insufficient viral RNA, it is unlikely during the peak of infection (Indik et al. 2005; Oleksiewicz et al. 1998). Instead, false negatives are more likely to occur due to a mismatch with the primer in the amplification region (Indik et al. 2005). Given the propensity of PRRSV to mutate and the developments towards precise and discriminatory RT-PCR tests (see below description on tests to detect highly pathogenic Type 2 PRRSV in Asia), ongoing efforts to ensure high diagnostic sensitivity are important (Kim et al. 2007).

Primer targets are selected according to the test purpose. Primers that amplify sequences that are conserved across genotypes and are resilient genetic change are ideal for detecting infection: Primers based on ORF7 and to a lesser degree OFR6 are commonly described for this purpose. Primers that amplify regions with hyper-variability are most useful to study genomic change (Indik et al. 2005; Oleksiewicz et al. 1998). Canadian diagnostic veterinary laboratories typically use primers based on ORF7 and ORF5. The ORF5 is prone to genetic mutation and so is most useful for understanding the spread of virus within and between herds. Diagnosticians ensure that all types of PRRSV will be detected by running a multiplex PCR (mPCR) reaction for Type 1 and Type 2; this is done by all the VDL in Canada that were contacted.

There are two common types of qRT-PCR multiplex reactions: Those using TaqMan® are more specific that those using SYBR® Green ®. With TaqMan® each target is bound to a unique probe that fluoresces a different color while the SYBR® Green quantifies the amplification of a single probe and subsequently quantifies each target by the melting point distribution (Balka et al. 2009; Lurchachaiwong et al. 2008). Several publications have compared different PCR protocols but given the narrow audience for such specific applications these were not summarized (Balka et al. 2009; Lurchachaiwong et al. 2008; Oleksiewicz et al. 1998).

All Canadian VDL offer multiplex PCR (mPCR) for Type 1 and Type 2 PRRSV but do not offer other mPCR reactions that include PRRSV. Reports of multiplex PCR (mPCR) tests include PRRSV combined with porcine parvovirus, porcine circovirus type 2, pseudorabies virus, and classical swine fever virus (Cheng et al. 2008; Jiang et al. 2010; Yang et al. 2006; Yue et al. 2009). Including FAD in PRRS monitoring is an interesting approach, given the similar clinical picture presented by many viral diseases.

Multiplex PCR tests have limitations. The analytical sensitivity can be negatively affected by including multiple primers and there are compromises in the optimization of reagents, annealing
temperature and cycling conditions with each pathogen added (Giammarioli et al. 2008). The relative sensitivity and specificity of the test as a singleton and combination reaction must be validated. Presuming that such logistical constraints could be overcome, mPCR technology has the potential to help the industry deliver a toolbox for endemic disease control and exotic disease surveillance. Concurrent monitoring and testing for several diseases could provide economies of scale. If foreign animal diseases were included, regular testing could alleviate concerns that an incursion could go unrecognized. This information would be of particular value to the CFIA given the use of such data in demonstrating continued disease freedom to international trading partners. Support for research on mPCR tests should be considered when developing a swine health surveillance system.

Highly virulent strains of PRRSV have caused severe and sudden outbreaks: Most dramatically in June 2006 a PRRSV outbreak occurred in China that caused high fever, morbidity and mortality. The responsible strain belongs to the Type 2 genotype (H-US-PRRS) but has 87 base pairs deleted from ORF2 resulting in a unique discontinuous deletion of 30 amino acids in nsp2 (Chen et al. 2009; Xiao et al. 2008). Currently, the classical Type 2 virus (C-US-PRRSV) and the highly pathogenic variant (H-US-PRRSV) coexist in much of south-east Asia (World Organization for Animal Health (OEI). 2010b).

Genetic evolution broadens the scope of questions that diagnostic tests must answer. In order to develop effective interventions, and understand the epidemiology, it is important to distinguish between infective strains. Detecting co-infection is also desirable as it can be a predictor for PRRS evolution. In the C-US-PRRS the sequence encoding for the unique nsp2 with the amino acid deletions in H-US-PRRS is not conserved so is not an appropriate target to differentiate these viruses. Rather a gene segment encoding for nsp3 with 2 nucleotide differences between C-US-PRRS and H-US-PRRS has been identified and used in a multiplex qRT-PCR (Chen et al. 2009). The authors acknowledge the concern of basing a test on a 2 nucleotide difference for a mutagenic virus. This example illustrates how rapidly diagnostic test needs can change and emphasizes the need for laboratory infrastructure that can rapidly respond to regionally specific needs.

REVERSE TRANSCRIPTASE LOOP MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP)

Traditional RT-PCR tests require laboratory expertise and specialized equipment. The advent of qRT-PCR has further increased the technicality and cost of the equipment. Access to specialized equipment and training is a limitation for animal health surveillance in countries like China (Chen et al. 2010; Chen et al. 2008; Cui et al. 2008; Qin et al. 2009; Zhou et al. 2009). This is less pressing in Canada, but submitting samples to a laboratory does involve shipping, handling, and delays results.

Loop mediated isothermal amplification (LAMP) is a form of nucleic acid amplification that can be done in a veterinary clinic or even on-farm. LAMP tests for RNA viruses use a reverse transcriptase step to convert RNA to cDNA and are referred to as RT-LAMP. The major advantage of RT-LAMP over RT-PCR is the ability to conduct the test with minimal equipment. While PCR reactions are dependent on the thermocycler to cause replication of the nucleic acid sequence,
LAMP amplifies nucleic acids under isothermal conditions that can be supplied by a simple water bath (Notomi et al. 2000).

Other less-known isothermal amplification approaches exist but have limitations due to poor specificity. The LAMP technique is highly specific because it uses four to six primers. This technique was first reported in 2000 for DNA (Notomi et al. 2000) and has been modified for the PRRSV RNA virus (Chen et al. 2010; Chen et al. 2008; Qin et al. 2009; Rovira et al. 2009). The basic premise is based on auto-cycling DNA synthesis and strand displacement. Concurrent with the DNA amplification process, a DNA polymerase with strand displacement activity displaces and releases a single stranded DNA (Notomi et al. 2000). The amplified product can be visualized in several ways. An undigested product can be visualized following electrophoresis onto an agarose gel. If it is undigested, a positive reaction appears as a smear because several primers were amplified. To increase specificity, the amplified product can be digested prior to electrophoresis and visualized as a predictable ladder. For field application the product can be visualized either through a change in turbidity or a color change if a dye has been added. Turbidity is more subjective but is preferred because it avoids opening the tube to add the dye which increases the potential for cross contamination (Rovira et al. 2009).

The earliest description of an RT-LAMP for PRRSV was applied to blood, semen and lung tissue and was combined with a commercial RNA extraction kit (Chen et al. 2008). The test was developed using a clinical H-US-PRRS isolate and targeted the ORF1a gene. A nested RT-PCR was used as the reference test. The RT-LAMP had a higher analytical sensitivity than the nested RT-PCR (5 copies/tube versus 25 copies/tube) and the reaction required 45 minutes. This report included unsubstantiated comments in the discussion and lacked robust test validation. However, as a preliminary investigation it demonstrated promise in the RT-LAMP approach.

A description by Qin et al described a test developed with six Type 2 PRRSV originating in China (Qin et al. 2009). The M gene was the target and outcomes were compared to RT-PCR and a qRT-PCR. There was 100% agreement between the RT-LAMP, RT-PCR and qRT-PCR for the 10 blood and 10 lung clinical samples tested. The analytical sensitivity of the test was reported as 10^{-4} dilutions, which compared to the RT-PCR at 10^{-2} and the qRT-PCR at 10^{-6}. These analytical sensitivity and specificity estimates should be considered preliminary given the small sample size. Total test time was 90 minutes, of which 45 minutes was for the RT-LAMP. The outcome was reported as both a gel-electrophoresis and as a direct visualization following the addition of Picogreen® dye. No information was provided on intra- or inter- laboratory test consistency. It is unknown how this test would perform on Type 2 PRRS originating from outside of China.

Chen et al. described an RT-LAMP targeting the ORF7 gene encoding the N protein (Chen et al. 2010). The test was developed using two Type 2 strains; a C-US-PRRS (CH-1a) and an H-US-PRRS (LXB-1) which is nsp2 deleted. A commercial kit (U-LAMP loop-mediated amplification universal kit, Mylab Corporation, Beijing) was used. An RNA extraction step was performed and RT-PCR and a qRT-PCR were performed as the reference tests. The analytical sensitivity was reported as 1x10^0 to 1x10^1 gene copies per reaction for both qRT-PCR and RT-LAMP while RT-PCR was 1x10^2. It was specific in that it did not amplify porcine parvovirus, porcine circovirus Type 2, pseudorabies virus, swine influenza virus, or classical swine fever virus. In clinical samples, the RT-LAMP was 100% correlated with virus isolation, and when compared to RT-PCR was 93.75% sensitive and 100% specific. The 78 field specimens were composed of sera
combined with tissues to ensure a high viral concentration but the authors note that sera alone could be used.

Rovira et al. developed a multiplex RT-LAMP for Type 1 and Type 2 virus (Rovira et al. 2009). A commercial RNA extraction kit was used. The test was developed using four well-characterized Type 2 field strains, two Type 2 attenuated vaccine strains, and one Type 1 field strain. It was validated on 114 samples from experimentally inoculated boars as well as 100 samples from known negative pigs. This study reported that the diagnostic test held promise. Favorable outcomes included that the results could be interpreted visually, either with or without an added dye, and that the reaction was highly specific. Unfavorable outcomes included finding the reaction was sensitive to the run-time duration, had variable analytical sensitivity across strains, had uniformly low analytical sensitivity relative to RT-PCR, and had only 43% diagnostic sensitivity.

The RT-LAMP is a promising technology but clearly requires more research. In principle a test based on 6 primers should be highly specific. However, this foundation also means it is challenging to ensure sensitivity across viruses from different regions and over time. The publication by Rovira et al was based on North American isolates and compared to qRT-PCR results from a North America laboratory reported unacceptably low sensitivity (Rovira et al. 2009). Once this test is refine enough to perform well under well controlled conditions, it will be imperative to investigate the reproducibility of RT-LAMP results under a wide range of field settings.

ANTIGEN CAPTURE ELISA

An antigen capture ELISA test has been developed in China to address the surveillance needs of identifying PRRSV infection without using RT-PCR (Cai et al. 2009) Limitations of RT-PCR based surveillance include expense, access to skilled labor and specialized laboratory equipment. The described test reacted to PRRSV N protein from Type 1 and Type 2 strains. It could be applied to sera or ground tissue as the specimen. When tested on 466 samples from vaccinated and symptomatic pigs under natural exposures, and compared to RT-PCR as a reference test, the sensitivity was 67%, specificity was 97% and accuracy was 93% (Cai et al. 2009). Currently there appear to be limited application for an antigen capture ELISA in North America given our access to cost effective and well validated RT-PCR tests.

ANTIGEN BASED IMMUNOCHROMATOGRAPHIC STRIP TEST (ICST)

The immunochromatographic strip test (ICST) technology was first developed in the 1980s for early at-home pregnancy detection in women. The tests works by binding the targeted substance to either an antibody or antigen that then binds to a protein labeled with colloidal gold. The substrate is applied at one end of a nitrocellulose matrix and diffuses through to the other end. If the target is present the bound complex forms a visible line and a control line. If the target is not present, only the control line appears. If the test fails, no lines appear (Achacha et al. 2010; Cui et al. 2008; Zhou et al. 2009). Immunochromatographic strip tests are ideal for on-farm testing because they require no special equipment or training, can be stored at room temperature for a relatively long time, provide an result quickly, and are low cost. The test cost is further reduced by eliminating sample handling and shipping (Cui et al. 2008; Zhou et al. 2009).
One antigen-based ICST has been described that targets the M and N proteins of PRRSV (Zhou et al. 2009). The test was evaluated using serum, whole blood, and tissue as specimens. Results were provided within 15 minutes. The test was stable for at least 30 days at room temperature prior to use. The test was applied to sera from 25 pigs experimentally infected pigs with a Type 2 strain LXB-6 and to 100 sera, bloods, lymph nodes and lungs from two naturally infected farms in China. Virus isolation, sequencing and RT-PCR were used as reference tests. The analytical sensitivity was between $7.8 \times 10^3$ to $1.6 \times 10^4$ TCID$_{50}$ ml$^{-1}$. From the 100 samples from the experimentally infected pigs, using the RT-PCR as the reference, sensitivity was 97% (65/67), specificity was 93.9% (31/33), and accuracy was 96%. Notably, virus isolation identified fewer positives than either the RT-PCR or the ICST which could either be attributed to a higher analytical sensitivity or the fact that virus isolation requires infective virus while the other two tests do not. The time to detection was similar to virus isolation or PCR where tests were reliably positive early in the course of infection with declining detection by 14 and 21 dpi. This test would require validation in with North America PRRSV and samples before it could be recommended. However, if such results were successfully described, it could be useful when used in conjunction with ICST for antibodies (see next section). An obvious potential application would be on quarantined gilts as immediate pen-side results would simplify animal movement decisions.

PRRSV INDIRECT TESTS

**ELISA**

Antibody tests are a mainstay of PRRSV diagnostics because of their cost and prolonged window of detection. Seroconversion to PRRSV occurs ~ 7 to 9 dpi and detectable antibodies remain for ~ 150 days (Batista et al. 2002; Lopez et al. 2004). Unlike PCR tests which are positive if any amount of virus is detected, antibody tests are qualitative. Despite the diversity of the PRRSV, there are antibodies that bind to stable, ubiquitous epitopes. The most commonly used antibody targets the N protein.

ELISA tests are reported as the sample to positive (SP) ratio where the positive is a known standard. A sample is interpreted as positive when the ratio exceeds a threshold selected to optimize both sensitivity and specificity. Both the dichotomous and quantitative results are useful to make inferences about PRRSV exposure but should be limited to group-level, not individual, interpretations given the substantial variation between individual pigs and between PRRSV strains (Kim et al. 2007). On a herd level, noting the covariates that predict a high response can help identify where PRRSV is circulating.

A variety of serological assays are available (World Organization for Animal Health (OEI). 2010b). IDEXX offers commercial tests that are the *de facto* gold standard in North America (Diaz et al. 2010). These tests are described because they are commonly cited as reference test. The IDEXX PRRS Ab tests detect antibodies specific to the N protein and detect exposure to Type 1 and Type 2 virus (Brown et al. 2009). These tests are periodically updated: Currently some veterinary diagnostic laboratories in Canada are offering the IDEXX 2XR Ab Test while others have converted to the updated IDEXX X3 Ab Test. When interpreting studies that use the IDEXX tests as a reference, the version should be considered given the ongoing improvements to test performance. Unfortunately, this detail is not consistently reported. The 2XR version has a
reported 97.4% sensitivity and 99.6% specificity. Field experience suggests that 1 to 2% false positives can be expected (Brown et al. 2009; Okinaga et al. 2009). The IDEXX X3 Ab Test claims 98.8% sensitivity and 99.9% specificity, which is 90% reduction in false positives over the 2XR. The primary application of the IDEXX tests are to identify pigs exposed to PRRSV: IDEXX tests cannot be used to differentiate between exposure to Type 1 or 2, cannot differentiate between exposures to natural infection versus vaccination, and cannot be used to infer protective immunity.

Tests detecting antibodies against antigen other than the N protein have been described. A recent study developed ELISA tests targeting antibodies against three ORF1 encoded proteins; nsp1, nsp2, nsp7 (Brown et al. 2009). The test that detected antibodies to nsp7 was most thoroughly described and was validated using the IDEXX ELISA 2XR Ab test as a reference test. The developed test had an overall sensitivity of 97.4% (95% CI; 94.4% to 99.1%) and specificity of 99.6% (95% CI; 99.6 to 99.9). The test was able to distinguish Type 1 and Type 2 infections. When applied to samples testing false-positive with IDEXX, 98% were negative (Brown et al. 2009). The key advantages of this test compared to the IDEXX Ab Test were the ability to distinguish between genotypes and the ability to assist with resolving unexpected positive results.

Antibody based tests that distinguish between natural and vaccine exposure enable veterinarians to monitor vaccine compliance and response, the prevalence of exposure to vaccine virus (both intentional exposure and reversion to virulence), and allow insight into the extent of protective immunity (Oleksiewicz et al. 2005). In practice, these tests are used in series: typically the sample is subject to an ELISA that determines if the sample contains antibodies to PRRSV, then distinguishes between antibodies to Type 1 or 2, then finally distinguishes between vaccine and wild type exposure. Two reports describe ELISA tests that can distinguish between immune responses to field and vaccine strains. A peptide ELISA based on antibodies against GP4 reportedly can distinguish between an immune response to Type 1 field and Type 1 vaccine virus (Oleksiewicz et al. 2005). No Type 1 modified live virus (MLV) vaccines are licensed in Canada; Unless Type 1 PRRSV becomes prevalent and vaccination becomes a strategy this tool has little Canadian application. The second study examined antibodies specific to nsp1 and nsp2 (Johnson et al. 2007). By using multiple nsp2 fragments, exposure to Inglevac MLVd could be distinguished from field virus infection. This paper did not validate the experimental test against a reference test.

Collectively these three reports illustrate potential developments for antibody testing. Continued research may result in tests that are complementary to each other and collectively address some of the epidemiological limitations of the IDEXX Ab Tests. Designing discriminatory diagnostic tests is not without challenges. Tests must be based on immunodominant antigens that induce a relatively consistent duration and amount of antibody across viruses, pigs, and environmental conditions.

**ANTIBODY BASED PEN-SIDE TESTS**

Four pen-side antibody tests that require minimal equipment or laboratory expertise claim to be technically simple, low cost and provide rapid results. All four report a dichotomous outcome, which is a notable difference from the ELISA tests. This simplifies on-farm interpretation but also eliminates some of the information and flexibility obtained from using the qualitative
interpretation for herd profiling or cut point optimization (Achacha et al. 2010; Cui et al. 2008; Lyoo et al. 2005; Mende et al. 2004; Mende et al. 2005).

The gel enzyme immunoassay (gel-EIA) was developed based on American PRRSV strains (Mende et al. 2004). The reported substrate was serum soaked filter papers though research is ongoing into using blood soaked filter papers. This test has only been described as a conference proceeding with limited details regarding its characteristics or validation. The gel-EIA provides results within 3 hours at 25 °C. Preliminary findings, using an IDEXX Ab Test as the reference, report 100% specificity and 93.7% sensitivity based on 150 samples. The limited description of test development and validation means these results must be considered preliminary (Mende et al. 2004; Mende et al. 2005). The authors state the primary intended application is timely determination of gilt-acclimatization success prior to entry into the sow herd.

Immunochromographic strip tests were described in the section on antigen tests. Three reports were found describing ICST that detect antibody. The BioSign™ PRRSV test detects antibodies to the N protein (Lyoo et al. 2005). The test uses serum and was developed and validated on Type 2 PRRSV strains from Korea and the United States. The publication does not state if the test detects Type 1 strains. Over 810 field samples were tested, of which 250 were from routine diagnostic submissions to the University of Missouri Veterinary Medical Diagnostic Laboratory. The BioSign™ was compared to the IDEXX PRRS Ab Test and a sensitivity of 98.7%, a specificity of 98.5% and an accuracy of 98.6% was reported. The discrepant samples were those with an ELISA SP ratio near the cut-off. Further, only 7 of 810 samples had an ambiguous outcome. These were tested twice and considered negative if the second test was also ambiguous. When applied to experimentally inoculated pigs, the BioSign™ and IDEXX PRRS Ab Test both sporadically detected exposure at 5 dpi and consistently detected exposure at 7 dpi.

The BioSign™ test has a strong potential for North American application. Routine diagnostic specimens were used to validate the test, which suggests minimal selection bias. Secondly, over 30% of the samples were from North America which reduces the concern of extrapolating sensitivity estimates between populations with dissimilar viruses. Finally, all isolates were tested with a commercial test which is currently used in Canada. This test has been submitted to the USDA for license approval, which was expected in 2010. Prior to use, it will be necessary to clarify it the test detects Type 1 PRRSV exposures.

A second immunochromatographic test strip differs from the BioSign™ in that it detects antibodies to both the M and N protein (Cui et al. 2008). It was developed using the LXB-1 Type 2 strain from China and was validated on 650 serum collected from pigs in China for diagnostic purposes. No other information was provided about these pigs to assist in determining if it is appropriate to extrapolate these findings to PRRS infected pigs in Canada. In this paper, the reference test was the CIVTEST™ SUIS PRRS, which is a commercially licensed test in Europe based on M antibodies (Mateu et al. 2006). Cui et al reported a sensitivity of 98.6%, a specificity of 97.8% and an accuracy of 99.3% when examined on field samples. Under experimental infection, the test had a sensitivity of 95.7%, a specificity of 98.2% and an accuracy of 96.8% with both the immunochromatographic strip test and the commercial ELISA detecting sporadic seroconversion at 7 dpi and consistent seroconversion at 10 dpi (Cui et al. 2008). The potential advantage of this test, over the BioSign™, is that antibodies against both M and N proteins are
used. The M protein is encoded by the most conserved region of the PRRS genome so in theory this test should operate under a wide variety of situations.

A third immunochromatographic strip test developed in Canada has been described in conference proceedings (Achacha et al. 2010). The test detects antibodies specific to the N protein. When evaluated against a commercial indirect ELISA and a western blot assay using serum from experimentally infected pigs, the sensitivity was 96.7%. The specificity based on clinical specimens was 99.3%. Communication with the company (AriVac Inc.) indicated the test target “all PRRSV strains worldwide”. This test has not been described in peer-reviewed literature and is not yet commercially available.

The advent of pen-side antibody tests is less groundbreaking than the pen-side antigen tests given the delay in time-to-detection for antibodies. Regardless, these tests appear close to market ready and once available will provide producers and veterinarians with rapid test results without the logistical issues of laboratory submission. The trade-off of a qualitative outcome will most likely mean that this type of test will be used in conjunction with, rather than replacing, ELISA tests.
DIAGNOSTIC SPECIMENS & COLLECTION TOOLS

Diagnostic specimens affect every aspect of diagnostic test performance and diagnostic results interpretation. For each specimen, a diagnostic test may require optimization to perform properly or account for differences in the presence, concentration, and availability of the analyte. The dynamic changes in the target over the course of the infection, compared to the traditional specimen, and the biological variability can affect when samples should be collected and how results are interpreted.

The logistics of diagnostic specimen collection can influence the sample size, sampling interval, and sampling frame of animals and farms. Specimens obtainable quickly and easily are likely to be collected regularly from more pigs. Specimens that sample a group rather than individuals, such as oral fluids, can increase sample size while decreasing collection cost. Specimens with stable storage or minimal handling requirements provide more opportunities to collect samples while still having quality material reach the laboratory. For PRRSV monitoring, the relevant interpretation of diagnostic test results is the herd level. Hence, diagnostic test sensitivity, specificity, sample size, pool size, testing interval, and detection prevalence all influence the confidence in PRRS status.

Disease prevalence does not affect test sensitivity or specificity. But, as prevalence increases the herd sensitivity increases because the probability of randomly sampling a positive animal from the population increases. This has implications for using diagnostic tests to monitor for disease-freedom. In order to achieve high herd sensitivity in a low prevalence situation, a highly sensitive test must be applied to many animals. Selecting the optimal sample size is a balance between improved herd sensitivity and declining herd specificity. Unless the test has perfect specificity, the more samples tested the higher the odds of a false positive. Larger sample sizes also cost more. Practitioners often wish to offset expenses by testing samples as pools.

General

Diagnostic test results are interpreted at both the sample and the herd level. When considering an individual specimen the test sensitivity is highest with no pooling because the analyte is not diluted by negative samples. When considering a herd, the sensitivity may be increased or decreased by pooling depending on the prevalence and whether the number of tests run is determined based on a probability of detecting a positive with a predetermined degree of confidence or on the number of tests that can be done with a fixed budget.

In situations where false negative results have serious consequences higher expenditures on diagnostic tests may be justified and pooling should be undertaken with great caution. In contrast, when disease status must be determined for the least cost pooling can provide a practical solution. The extent to which samples can be pooled is dictated by the analytical sensitivity. The extent to which samples should be pooled is influenced by the consequence of a false negative. Further, if the individual samples contributing to a positive pool will be retested to confirm the result the most cost effective pool size (x) is affected by disease prevalence (\(\rho\)) (Figure 1) \((\rho < 1 - x \sqrt{1/x})\). (Salman. 2003).
Figure 1. Maximum prevalence that a pool of a given size is cost effective assuming the samples contributing to the pool are retested individually in all positive pools.

PRRSV Direct Tests
Boar studs commonly pool serum for RT-PCR testing. Using experimentally inoculated boars, Rovira et al compared the sensitivity of RT-PCR on serum in pools of 3 and 5 with one positive sample per pool (Rovira et al. 2007a). Over the first 2 weeks of infection, the difference in the number of positives detected from individual samples compared to pools of 3 and 5 was never greater than 1/18 boars. Across the time points, ~6% of the samples that would have been detected as positive if tested individually would be missed by testing serum in pools of 5. Compared to what would be expected from field samples, this could be considered a worst case estimate because only one positive was included in each sample (i.e. mimicking a low prevalence situation) and a mild isolate was used which would typically produce fewer viral copies than a virulent isolate.

Sow herd stability is commonly defined as weaning virus negative pigs and is determined by testing serum with RT-PCR. Compared to an assumed perfect sensitivity on individual samples, RT-PCR on pools of 5 serum had a sensitivity of 84.5% (95% CI; 73.5 to 91.6%) and on pools of 10 had a sensitivity of 82.0% (95% CI; 68.1 to 91%). Despite this decrease in sensitivity, it was noted that if 60 piglets were tested per farm the case sensitivity and specificity was 100% regardless of if samples were tested individually, as pools of 5, or pools of 10 (Polson et al. 2010). Assuming $8.00 per PCR test means the relative cost of these protocols would be $480, $90, and $48 respectively.

PRRSV Indirect Tests
In contrast to pooling serum for PRRSV RT-PCR, most Canadian veterinary diagnostic laboratories discourage pooling serum for PRRSV ELISA testing. This stance has been made because of concerns of dilution affecting analytical sensitivity. These concerns are justified at the test level but can be minimized by optimizing the threshold and have less effect when the herd sensitivity is the outcome of interest.

A French study tested two unnamed but commercial ELISA assays on pooled sera (Hutet et al. 2003). Both performed similarly and will be described together. An a priori decision established that a true positive pool needed at least a 40% chance of testing positive. Hence a pool size of five was chosen because the average antibody titres resulted in a pool of 5 with a single positive having a 40 to 50% chance of testing positive and a pool of five with two positives having a
>80% chance of being testing positive. When pools of five were compared to individually tested samples, the sensitivity was 71% and the specificity was 100%. The recommendation from this paper was that the current protocol of sampling 7 sows and 5 grow-finish pigs be replaced with 15 samples pooled into 3 tests of 5. The sensitivity of this strategy was 89% compared to the individual samples but reduced testing costs by 75%; funds were reallocated to other control and eradication activities. This study also tested pooled filter paper elutions as described in that section.

A second paper further supports the premise of ELISA tests on pooled serum. The pool sensitivity was investigated for pools up to 10 sera containing only one positive per sample. For pools of 4, a pool-sensitivity of 0.69 was reported for a SP cut-off of 0.4. However, a cut-off of 0.2 had a pool sensitivity of 0.84 while also minimizing false positive (Rovira et al. 2008). The benefits of pooling are most pronounced when the outcomes are herd sensitivity and specificity assuming a fixed budget. Under this situation, testing 10 individual samples with ELISA results in a herd sensitivity of 0.67 and herd specificity of 0.85 while pools of 4 or more had a herd sensitivity of >90% and herd specificity >80%. These estimates are conservative as this paper assumed a herd prevalence of 10% and only included 1 positive serum per pool (Rovira et al. 2008).

Pooled serum for RT-PCR testing is already an industry standard while uptake of pooled ELISA testing has been slower. Clearly, ELISA testing on pooled serum causes discomfort to diagnosticians who worry that effects on analytical sensitivity will not be considered in result interpretation. Yet, peer reviewed publications support this approach. The take home messages are as follows: First, pooling should be done very cautiously in situations where false negatives have severe consequences and the cost of individual testing is justified and; Second, that the pool size and number of pools tested should be selected to optimize the herd sensitivity and specificity while accounting for the budget.

Capillary Tubes

PRRSV Direct Tests
Blood collection from the auricular vein rather than jugular vein is common in boars that are subject to regular sampling (Broes et al. 2007). This technique is safer for handlers and reduces stress on boars because it does not require restraint. Ear veins are relatively small and not conducive to Vacutainer™ blood collection so swabs and capillary tubes are common collection vessels. Patterson et al followed 30 experimentally exposed pigs and found equivalent RT-PCR results for serum extracted from blood collected by jugular venipuncture and for blood collected by capillary tube in the first 4 weeks post infection (Patterson et al. 2007).

Several capillary tubes are commercially available including the Safe-T-Fill Serum Gel Capillary Collection, Microtubes, and Bio-Tubes. The main difference between these appears to be the volume of blood collected (300 – 600 μl) and harvestable serum (200 – 450 μl) (Broes et al. 2007). Ideally, tubes should be sufficiently large to allow a RT-PCR test, a follow up RT-PCR test, and an ELISA test. Theoretically, there is no reason that blood from capillary tubes could not be tested as a pooled sample. A practical limitation may be having an insufficient volume to retest when necessary or to determine which sample in a pool was positive.
**PRRSV Indirect Tests**

Although several capillary tubes are available, only a single study was found describing the effect of capillary tube samples on ELISA tests. The effect of the SAFE-T-FILL on the sensitivity and specificity of the IDEXX 2XR PRRS Ab test was examined in experimentally exposed pigs (Patterson et al. 2007). The sensitivity of samples in weeks 2 to 7 post infection was 100% for both jugular blood samples and capillary tubes when the manufacturer recommended cut off was used.

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**BLOOD SOAKED SWABS**

**PRRSV Direct Tests**

Blood can be collected from the ear vein of boars with a swab. Swabs have the potential to be less sensitive than serum because a smaller volume of blood is collected and it is further diluted in saline (Reicks et al. 2006). This has been demonstrated in an experimental inoculation trial where virus concentration was consistently and statistically significantly lower in swabs than serum (Rovira et al. 2007a; Rovira et al. 2007b).

The sensitivity and specificity has been compared for RT-PCR on swabs versus serum in boars. All studies have found this technique to be highly sensitive. Patterson et al followed 30 experimentally exposed pigs (7 to 8 weeks of age) and found equivalent results for samples collected by jugular venipuncture and swab for the first 4 weeks post infection (Patterson et al. 2007). Rovira et al found individual blood swabs and serum had virtually identical sensitivity in 18 boars traced from exposure to 15 dpi (Rovira et al. 2007a). Reich et al found that serum and swabs were 90% correlated at 24 hours (90% CI: 61 to 99.5%) and 100% (90% CI: 74 to 100%) correlated thereafter (Reicks et al. 2006).

Boar studs often monitoring for PRRSV with pooled swabs tested with RT-PCR. Using experimentally inoculated boars, the sensitivity of RT-PCR on serum and swabs tested individually, swabs as pools of 3 (1 positive and 2 negative), and swabs as pools of 5 (1 positive and 4 known negative) were compared (Rovira et al. 2007a). Compared to serum from individual boars, an 8% decrease in sensitivity was found for swabs pooled 1:3. Compared to swabs from individual boars, pools of 3 had slightly lower sensitivity in the first 5 dpi (detected between 1 and 3 fewer positive boars out of 18 studied) and pools of 5 had slightly lower sensitivity for the first 10 dpi (detected 1 less boar in the 18 studied) which is likely explained by a dilution effect the virus concentration was consistently and statistically significantly lower in swabs than serum (Rovira et al. 2007a).

Extrapolating the finding by Rovira et al. to field application has raised concerns. Under the carefully controlled conditions sensitivity was minimally affected by pooling. However, swabs from field samples may be less thoroughly soaked in blood or more dilute if the ratio of saline to blood is too high. In theory this would lead to a higher rate of false negatives than reported. These concerns are justified but the study design of including only one positive per pool and using a design prevalence of 10% mean that the estimates in this paper are conservative.

**PRRSV Indirect Tests**

The concerns that blood swabs are a small, diluted sample with inherent limitations for analytical sensitivity are more pronounced for ELISA testing than RT-PCR (Patterson et al. 2007). On a
practical level, ELISA testing on any sample is discouraged in studs because of an unacceptable
time to detection (Rovira et al. 2007b). So for samples from boars, the possibility of decreased
sensitivity on ELISA is mainly relevant because of limitations in using ELISA as a follow-up test
for questionable RT-PCR results. The ELISA sensitivity with swabs may become more relevant if
other classes of pigs are sampled with this technique.

The effect of a polyester swab on the sensitivity and specificity of the IDEXX 2XR PRRS Ab test
was examined in experimentally exposed pigs (Patterson et al. 2007). The sensitivity of jugular
samples in weeks 2 to 7 post infection was 100% while swabs ranged from 20 to 56% when the
manufacturer recommended cut off was used. Optimized cut-offs from weeks 2 to 7 post
infection were much higher for jugular venipuncture (0.82 to 1.54) compared to swabs (0.02 to
0.15). When the optimized SP ratios were applied, sensitivity was 100% for weeks 2-7 and
specificity was 100% for weeks 3-7.

Practitioners wishing to use swabs for ELISA testing should work closely with their diagnostic
laboratory. The cut-off should be carefully considered as well as the ability to collect consistent
specimens from the field. At this point, pooled swabs for ELISA testing cannot be recommended.

FILTER DISCS

General
Serum is the mainstay for PRRSV testing. However, venipuncture poses concerns for animal
welfare, worker safety, and requires experience. Serum must be separated from the whole blood
components, which requires labor and creates opportunity for sample cross contamination.
Serum must be stored under refrigeration temperatures for immediate testing or frozen for
longer term storage. Shipping serum involves transporting potentially infectious virus.

Filter paper is a cellulose-based matrix that usually contains chemicals to lyse cells while
preserving nucleic acids. It addresses many of the sampling issues posed by serum and can also
be used for a wide range of other specimens including whole blood (with or without
anticoagulant), serum, post-mortem tissue imprints, and oral fluids (Inoue et al. 2007;
Torremorell et al. 2011). When used for serum or blood, a small volume is needed so many blood
collection sites including ear and tail veins are appropriate.

Filter papers have robust handling characteristics. Pathogens are denatured and non-infectious,
so nucleic acids can be detected but virus cannot be isolated (Torremorell et al. 2011). The
nucleic acids are protected from nucleases, oxidation and UV damage and are stable for long
periods of time at room temperatures (Inoue et al. 2007). A comparison of the RT-PCR sensitivity
on FTA cards spotted with blood, post mortem tissues, or oral fluids found no differences in RT-
PCR sensitivity after storage for 14 days at either 4 °C or 25 °C compared to immediate testing
(Dokland. 2010; Torremorell et al. 2011).

For diagnostic testing, a punch is used to remove a sample-soaked disc of known size from the
filter paper. This is placed in solution and the elution is applied to the diagnostic test. Several
different filter papers are available. The compositions differ and are proprietary. Publications
generally report which paper was used and the following three were considered in the
veterinarian survey associated with this report TEGOTM, FTA® m, and GeneSeek® h. The FTA and

- 21 -
GeneSeek® papers were developed for use in genomics with extension into diagnostic. The TEGO™ was developed as a diagnostic test media.

**PRRSV Direct Tests**

RT-PCR tests conducted on filter disc elution have a lower analytical sensitivity than when run on serum because of sample dilution during the elution process. A 10-fold decrease in analytical sensitivity was reported for serum soaked filter disks ($10^1 \text{ TCID}_{50}\text{ml}^{-1}$) compared to serum ($10^0 \text{ TCID}_{50}\text{ml}^{-1}$) (Linhares et al. 2010b). When RT-PCR was applied to the TEGO™ Animal Blood Collection (ABC) kit, qualitative results were comparable between serum and filter papers but lower Ct values were reported. The TEGO™ ABC was used with field samples and compared the routine diagnostic procedure at Iowa State University Veterinary Diagnostics Laboratory. From this a 93.8% sensitivity and 99.5% specificity was reported (Yoon et al. 2010).

A comparison of the sensitivity of three available filter papers has been presented but the dataset included only 31 positive sample derived from diluted vaccine virus in whole blood and 15 negative samples. The preliminary estimates of were as follows: TEGO™: 58% sensitivity, 92% specificity; FTA®: 74% sensitivity, 57% specificity; GeneSeek®: 55% sensitivity, 78% specificity. These estimates must be considered preliminary until the full report is released but will be valuable in determining if these products can be treated as a single “class” when making sampling recommendations or if brand specific recommendations will be needed (Abbott et al. 2011).

In theory, filter papers could be used as a storage media for oral fluids. This option combines the benefits of inexpensive pen-level sampling with robust handling and storage (Linhares et al. 2010a; Linhares et al. 2010b; Torremorell et al. 2011). An analytical sensitivity between $10^3 \text{ TCID}_{50}\text{ml}^{-1}$ and $10^5 \text{ TCID}_{50}\text{ml}^{-1}$ was reported for oral fluids on filter discs compared to $10^1 \text{ TCID}_{50}\text{ml}^{-1}$ for serum on filter discs (Linhares et al. 2010b). This makes biological sense given that oral fluids have a lower viral concentration than serum. What was less expected was that the sensitivity of filter discs with oral fluids appears to decline over the course of infection in a manner that is not consistent with the decline in oral fluids without filter paper. By 19 dpi, OF stored on FTA papers were an unreliable diagnostic specimen (Torremorell et al. 2011). Further work is required to understand the reasons for this. Recent work has described optimization techniques for RT-PCR on oral fluids (Chittick et al. 2011). Similar work will be needed if the oral fluid / filter disc combination is pursued given that contradictory information has been published regarding the need for a pre-processing step. Linhares et al. required a lysis solution of isothiazolon (Kathon DC) at 3 ppm while Prickett et al. found this was of no value (Linhares et al. 2010b; Prickett et al. 2010).

**PRRSV Indirect Tests**

Two reports were identified describing filter discs for PRRS antibody testing. The TEGO™ ABC has an elution process has been optimized to allow both an ELISA and RT-PCR to be run on the same disk. An experimental exposure study compared ELISA tests on serum and filter disc elution from samples collected weekly. For Type 2 exposures, PRRSV, antibodies were first detected at 7 dpi in serum and 14 dpi using filter discs. For Type 1 exposures, PRRSV antibodies were detected at 14 dpi for both serum and filter discs. When antibodies were detected for a sample type, the majority of samples were positive. As expected, lower SP ratios were reported with filter disc elution compared to direct testing on serum (Yoon et al. 2010).
A second study investigated the use of pooled filter disc elution for ELISA testing to reduce the cost of serological diagnosis for regional monitoring. The type of filter paper and the two ELISA kits were not reported but were commercially available (Hutet et al. 2003). This study has been described in more detail under the pooled serum section. For the filter discs, an optimized cut off was established based on the average result plus three standard deviations. Paired filter disc and serum samples, were titrated in two-fold serial dilutions and resulted in similar titres. The sensitivity of individual filter discs compared to individual serum was not reported. Instead, the sensitivity of individual filter discs was compared to pools of 5 filter disks; with a 79% sensitive and 86 to 97.5% specificity. This large range in specificity is most likely to do a flawed trial design with the upper end representing the true value. Inter-laboratory agreement was satisfactory when tested on eight regional labs. From these results, it was recommended that serological monitoring for PRRSV could be conducted using either pooled serum or pooled filter discs. The main limitation of applying these results was the failure to compare individual filter disc and serum results so all changes in sensitivity are relative to the sensitivity of filter discs, which in Canadian laboratories is currently unknown.

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**Meat and Meat Transudate**

### PRRSV Direct Tests

Successful national or regional control of PRRS will require cost-effective, timely diagnostic samples to detect virus circulation in an unbiased sample of the population. This creates challenges because herds with high-risk practices may be biased to non-participation. One option is to collect diagnostic specimens at abattoirs. This sampling point does not have the biosecurity problems associated with on-farm collection. It also provides easier and less expensive access to an unbiased sampling frame, which enables valid PRRSV prevalence estimates. There is a precedent for abattoir-based monitoring of reportable and non-reportable diseases in Canada (Canadian Food Inspection Agency. 2009; Government of Canada. 2010). It remains unclear who can legally provide permission to sample and test pigs at slaughter.

An abattoir based sample is muscle transudate, also known as meat juice. Meat juice is the fluid that is recovered from frozen meat as it thaws. Meat juice has been used in a Canadian slaughter-based testing (Larochelle et al. 1997; Magar et al. 1995; Magar et al. 2004). Serum and meat from over 1000 Canadian pigs at slaughter were tested for PRRS specific antibodies, nucleic acids, and virus (Magar et al. 2004). Most samples (74%) were seropositive but only 4.3% of serum samples and 1.9% of meat samples were PCR positive. This finding has implications for exporting pork to PRRS-naïve areas, as some PCR positive samples were able to cause infection in naïve pigs exposed orally to the meat. For the purposes of this diagnostic test review, the more relevant finding is that while most pigs have antibodies persisting until market they do not have detectable virus so abattoir based surveillance is appropriate to determine prevalence but inappropriate to track case incidence.

### PRRSV Indirect Tests

A comparison of diagnostic test performance for three antibody detection tests on serum and muscle transudate has been reported. The tests considered were the IDEXX 2XR, an indirect fluorescent antibody test (IFN), and a fluorescent focus neutralizing (FFN) assay. Given that the ELISA is the only test with application for large scale monitoring these results were focused on. The study reported 100% specificity. The sensitivity could be optimized to meet or exceed the
sensitivity on serum by adjusting the specimen/dilution ratio and the optimal SP cut-off. Dilutions of 1:5 were ideal because coagulation was a problem with lower dilutions. This dilution was optimized at an SP ratio of 0.034 (sensitivity = 96.7 (95% CI 90.6 to 99.3)) but this was too close to zero for practical application. Therefore, the recommendation was to consider a cut-off of 0.1, 0.2, or 0.3 and adjust sensitivity accordingly.

The herd sensitivity of ELISA on meat juice was modeled for a population of 1000 animals based on 5 or 10 samples across a prevalence range of 0 to 40%. If 10 samples were tested per population, and an ELISA SP threshold of ≥0.1 was used, the probability of misclassifying a negative herd was 5% while the probability of correctly classifying a herd with 10% true prevalence was 66%. (Molina et al. 2008). Denmark has successfully conducted PRRSV monitoring using meat juice samples at slaughter.

**MILK**

**Genome & Antibody Detection**

The PRRSV and antibodies against PRRSV are present in milk (Kang et al. 2010). Little work has been done on the feasibility of using milk samples for PRRS monitoring, and given the practicality of collecting milk this may be of little interest. However, it is a sample that can be collected with no negative welfare for on the sow. Theoretically, milk may be a diagnostic specimen for PRRSV testing.

**Neonatal Tissues**

**PRRSV Direct Tests**

Transplacental PRRSV infection can be monitored using serum from live-born presucked piglets, thoracic fluid from dead piglets/mummies, or tissues form dead piglets/mummies for RT-PCR. Tissues or thoracic fluid can be pooled up to 1:8 for RT-PCR testing with no reported loss of sensitivity. This allows an entire litter to be tested with one to two RT-PCR tests. In a small study, the RT-PCR sensitivity on tissues held at temperatures up to 37 °C for up to 96 hours were minimally affected. This supports the use of autolized and mummified samples from aborted fetuses (Benson et al. 2002).

**PRRSV Indirect Tests**

Serology on pre-suckled neonates and dead piglets is an insensitive test. Experimental exposure of sows during the window of piglet immunocompetence, paired with IFA testing of near term piglets with a homologous IFA test, found 15% of congenitally exposed piglets had detectable antibody. This corresponded to half of the examined litters having no seropositive piglets. The use of serology in piglets at birth was not supported (Benson et al. 2002).

**Oral Fluids**

**General**

As early as 1901, researchers in human and veterinary medicine recognized that the same ‘principles’ could be detected in serum and saliva. More recently, a report of HIV antibodies in oral fluids reignited interest in oral fluids as a diagnostic specimen (Archibald et al. 1986; Prickett et al. 2010). Oral fluids were first reported as a diagnostic specimen for PRRSV in 1997 (Wills et
al. 1997). This review is limited to PRRSV, but it is noteworthy that oral fluids samples have diagnostic value for many diseases. A review by Prickett et al. noted that work has been conducted with oral fluids and classical swine fever, vesicular stomatitis virus, foot and mouth virus, *Actinobacillus pleuropneumoniae*, and PCV2 (Prickett et al. 2010). The true value of oral fluids may be realized through multiple pathogen surveillance.

Oral fluids are a mixture of saliva and mucosal transudate. Saliva is mostly water containing enzymes that begin food digestion. The transudate the from capillaries in the oral mucosa and gingiva mirrors the presence and concentration of serum antibodies, hormones, drugs, and viruses. (Prickett et al. 2010). Passive transudation of serum components into oral fluids is extremely rapid resulting in a strong correlation between time to appearance of virus and antibodies in serum and oral fluids. From a diagnostic perspective this is important; other diagnostic specimens such as semen have been largely abandoned due to a delay in time-to-detection of virus (Rovira et al. 2007b).

Not all specimens collected from the oral cavity are equal. The composition is influenced by the method and site of collection. This review defines oral fluids as the fluid in the oral cavity collected by use of an absorptive device. In pigs this is most easily achieved by providing a cotton rope for them to chew on (Prickett et al. 2010). This approach is simple, rapid, inexpensive and non-invasive. In this review buccal and tonsilar swabs are not considered oral fluids. Buccal swabs have been evaluated and found to have a poor sensitivity when compared to serum tested with RT-PCR (Prickett et al. 2008b). Given the minimal research on buccal swabs and the lack of promising results, this specimen was not examined further. Tonsil swabs in pre-weaned pigs are considered briefly in the subsequent section.

All classes of pigs will readily donate oral fluids via ropes. Procedures have been refined to reflect the physical, behavioral, and husbandry differences between age groups (Prickett et al. 2010a; Thomas et al. 2011). Ropes are usually hung for 20-30 minutes, which is long enough to ensure many pigs interact with the rope and not so long that the oral fluids evaporate. In general, the rope and pig size are correlated with a recommended rope diameter of 1.3 cm for nursery pigs and 1.6 cm for grow-finish pigs and boars. Ropes should be hung at shoulder height. By forcing pigs to reach up slightly, sample contamination is minimized. Unpublished observations by Dr. Jeffery Zimmerman (Boehringer Ingelheim Meeting, Chicago, IL, Dec 6, 2010) note that ropes can be used to collect oral fluids samples from suckling piglets but the rope should be hung where the sow can touch the rope to increase piglet interest and willingness to chew.

The number of pigs that interact with the rope affects the sensitivity and probability of virus detection in a low prevalence population. Thomas et al investigated pig interactions with a rope in pigs 1.7 days post weaning (Thomas et al. 2011). Two ropes were hung per pen and the oral fluids pooled to make a single pen sample. In traditional nurseries with 24 pigs per pen, the participation rate was 91.3% (95% CI 89.5 to 93.1%). In wean to finish nurseries stocking 52.6 pigs per pen the participation rate was 40.3% (95% CI: 37.1% to 43.5%). Despite the lower participation rate in wean to finish nurseries the number of pigs interacting with the rope was similar suggesting a competition effect.

Oral fluid sampling in boars has been described (Kittawornrat et al. 2010a; Kittawornrat et al. 2011; Kittawornrat et al. 2010b). Seventy of 72 boars ranging from 6 months to 3.6 years learned to readily chew on a rope within one week. A flavoring solution (unsweetened apple juice
with sucrose) was used, which did not appear to affect sensitivity but was not formally examined. Boar stud sampling protocols should consider the risk of never sampling boars that fail to participate; traditional collection methods may still be required on these animals.

Kittawornrat et al also determined that infection with a mild strain of PRRSV did not affect the success rate of oral fluid collection but did lower the amount of fluid collected. An average of 17.6 ml (range 4 to 38 ml) was collected pre-infection and an average of 15.7 ml (range 1 to 37 ml) post infection (Kittawornrat et al. 2011). Given that an optimal amount of oral fluid is 4 ml, and all collections reportedly produced sufficient oral fluids for PRRSV qRT-PCR testing, it appears that mild PRRSV infections have little likelihood of resulting in insufficient sample to test (Prickett et al. 2010a). Presumably severe infections would be identified by clinical signs rather than routine monitoring.

Collection ropes should be cotton because it is highly absorbent (Prickett et al. 2010a). Ropes reportedly must not be bleached (Personal communications, Stephanie Norell, TEGOTM and Rick Bergmann Paradigm). It is unclear how failing to follow these recommendations would affect diagnostic test performance but ropes are readily available to producers and it is possible that changes to the type of rope could cause false negative test results without raising suspicion. To avoid this problem, clear communication between producers, veterinarians and veterinary diagnostic laboratories will be required. Oral fluids samples with visual particulates should be centrifuged prior to submission and testing (Prickett et al. 2010a). Diagnosticians have commented that oral fluid samples appear highly variable; some being very clear and others flocculent. This may be exacerbated by inconsistent centrifuging of samples. The effect of sample quality or consistency on sensitivity has not been reported. This information would be valuable for estimating reliability. Further research on the effect of rope type, rope treatment, and centrifuging on test results would be of value.

Once the rope is removed from the pen, it is wrung into a plastic bag and the fluid drained into a tube (Falcon 2054 or equivalent). Without careful handling, these steps could cause cross-contamination of specimens. This is not a concern for herd level diagnosis but could falsely elevate the within herd prevalence estimate. Fluids that will be analyzed within 24 hours should be chilled, while those with delayed testing should be frozen (Prickett et al. 2010a). Over a 12 day observation period, the highest virus concentrations and SP ratios were found in samples held below 10 ºC, an intermediate result at +20 ºC and the poorest results at +30 ºC. Between -20 ºC and +10 ºC no significant difference were found (Prickett et al. 2010).

PRRSV Direct Tests
The viral load in oral fluids is correlated with, but lower than, in serum (Kittawornrat et al. 2010a; Prickett et al. 2008b; Shah et al. 2011; Shah et al. 2011). Lower viral concentrations were found in oral fluids at days 0, similar levels at 14, and higher levels in oral fluids at 21 dpi compared to serum. Along with significant differences in viral concentration between specimens (serum versus oral fluids), differences have been found between PRRSV strains (MN-184, D09-012332, and Ingelvac PRRS MLV®) with interaction between the two. When the effect of onset, duration, and magnitude of viral shedding were accounted for by comparing the area under the curve (AUC) for viral concentration in serum and oral fluids a correlation of $r^2=0.68$ was reported (Kittawornrat et al. 2010a).
When using this information to interpret the interpretation of diagnostic test results, it is useful to keep in perspective that the difference of PRRSV concentration between strains was as large as the difference between oral fluids and serum (Kittawornrat et al. 2010a). In the field, additional variation may be induced by partial immunity, age, multiple viral strains and variable collection times post infection. From one perspective it could be argued that more research is required because failing to account for this variation might overinflate sensitivity estimates. On the other hand, natural variation has always been present in our diagnostics and we have not previously delayed access to diagnostic tests until all field variation is explained.

Oral fluids have been promoted as a screening tool to identify early PRRSV infection. This means sensitivity is affected by both the lower concentration of virus in oral fluids and potential dilution of a few positive animals in a sampling pool of 20 or more pigs. When a single viremic pig was placed in a pen of 24 other animals the probability of detecting PRRSV in that pen 14h after pen-mates were exposed to the viremic pen was 0.667 (95% CI, 0.49 to 0.81). This is substantially higher than would be expected under a serum-based monitoring protocol sampling 5 pigs per pen (5/26=0.192). To achieve a similar sensitivity 17 of 25 pigs would need to be bled (Wang et al. 2010). Further, 77% of the oral fluids samples collected 2 to 8 days after the viremic pig was placed in the pen were positive. This study demonstrated the value of oral fluids as an early detection monitoring tool, and also illustrated the value of using oral fluids to sample over time rather than as a point in time estimate (Wang et al. 2010).

The likelihood of early PRRSV detection should be confirmed through observational trials because the findings of Wang et al. were not fully corroborated in an on-farm trial (Prickett et al. 2008a). Three herds were studied by collecting 1 oral fluid and 5 blood samples from 6 pens at 3 week intervals. In two of three herds, PRRSV was detected in the majority of pens by serum and oral fluids on the same sampling visit. But, in one of the 3 herds the first visit with PRRSV detected was characterized by all 6 pens positive on serum qRT-PCR and all 6 negative on oral fluids. Time to detection of PRRSV in oral fluids appears to be similar in oral fluids and serum. Virus was detectable in 10% of boars within 1 dpi, 76% within 2 dpi and 94% within 3 dpi which was comparable to the 100% positive serum samples at 3 dpi (n = 70) (Kittawornrat et al. 2010b). Interestingly, significantly higher proportions of oral fluids samples remained positive at 14 and 21 days than serum samples suggesting that the window for detection may be slightly longer for oral fluids than serum (average over trials: Oral fluids 94% versus serum 80% at 14 days and oral fluids 90% vs. serum 79% at 21 days). As noted under filter discs, this finding may be dependent on how the sample is tested as oral fluids on filter discs were unreliable by 19 dpi (Torremorell et al. 2011).

The nucleic acid extraction procedures and PCR protocols to optimize the sensitivity of RT-PCR testing of oral fluids is ongoing. A preliminary report listed on-going work that included testing serial dilutions to describe the linear amplification range, pooling experiments to describe the detection limits within pens, and the ability to detect a one strong and weak positive pig per pen respectively (Munoz et al. 2010). PCR inhibitors exist in oral fluids and feces (Prickett et al. 2010). With the rope collection method, samples are commonly tainted with feces and other environmental contaminants. A comparison of 5 extraction procedures and two PCR protocols has been published (Chittick et al. 2011). The extraction procedures and PCR protocols were all based on commercial kits with deviations from instruction sufficiently reported for other
laboratories to be able to implement the protocols. Across all protocols combined the specificity was 98.4% (based on 430 samples). The protocols had significant differences in sensitivity with one protocol being significantly better than 7 of the remaining 9 (range 17 to 56%). Overall, using a 2X master mix which used twice the enzyme reduced the failure to find the internal positive control and reduced Ct variation for every extraction protocol over a 1X master mix. These results will assist laboratories in optimizing PCR testing on oral fluids but more work will be needed to determine the optimal protocol given the substantial variation in detection limits between strategies.

PRRSV Indirect Tests
Evidence supporting ELISA testing for PRRSV antibodies in oral fluids is limited and preliminary. Prickett et al reported no significant difference in ELISA values between oral fluid samples collected from experimentally inoculated pigs and controls (Prickett et al. 2008b). More recently three conference proceedings involving some of the same researchers have indicated ELISA tests can be used. One noted that the IDEXX 2XR Ab Test can be adapted to oral fluids to achieve a diagnostic sensitivity of ~80% and diagnostic specificity of ~100% (Prickett et al. 2009). A second reported that PRRS-specific IgG, IgM, and IgA isotopes in oral fluids can be identified (Prickett et al. 2010b). The third described samples tested at the Iowa State University Veterinary Diagnostic Laboratory. Samples were collected from two "high-prevalence" and two "low-prevalence" herds, where prevalence was undefined. In each herd, 3 age-groups had 6 pens sampled each (Dufresne. 2011). In the high-prevalence herds where the ELISA was reported, a diagnosis based on 5 serum samples per pen or one oral fluid sample were correlated between 50 and 100%. The average serum SP, average rope SP, and pen correlation all increased with age from 6 to 8 to 10 weeks. Few positives were reported in the low prevalence sites.

It appears that ELISA tests on oral fluids have been developed by the Iowa State University Veterinary Diagnostic Laboratory, as this test is commercially offered (Prickett et al. 2010a). Researchers affiliated with this institution have a strong record for publishing diagnostic test validation studies and are responsible for the four publications cited above. Hence, more detail regarding test optimization will presumably be coming soon and may include items they have noted previously such as changes to incubation times, sample dilution, detection threshold.

**TONSIL SWABS**

PRRSV Direct Tests
Tonsil swabs, while originating from the oral cavity, should not be considered oral fluids. Instead, this specimen is a non-invasive alternative to blood collection from pre-weaned pigs. Sow herd stabilization is commonly defined as negative RT-PCR results on pre-weaned pigs. A swab with a polyester-tipped applicator placed into a falcon tube with 1 ml of phosphate buffered saline was compared to serum with 100% concordant results when examined on a known positive population (n = 10), known negative population (n = 10) and a field situation (n = 180) (Schlueter et al. 2011). This study was based on a small number of samples so before being considered field ready, studies will be needed on more field samples and the specificity will need to be established on a much larger population. Oral fluid collection with ropes has also been described in this age group (unpublished description, Dr. Jeff Zimmerman, Boehringer Ingelheim Meeting, Chicago IL, Dec 4, 2010), which provides a less labor intense option, but tonsil swabs
may remain of interest given practitioners report it is challenging to get sufficient saliva from pre-weaned pigs (personal communication, Dr. M. Benjamin, Vets, Alberta).

**PRRSV Indirect Tests**

No reports of tonsil swabs for PRRSV antibody testing were found.
SUMMARY

The most promising outcome of this review was identifying the variety of novel approaches and tests for monitoring PRRS. An exponential increase in studies on alternative testing approaches has occurred over the last 5 years suggest. Many are promising and suggest we are on the cusp of marked changes in PRRS monitoring. Decisions regarding animal entry or mixing will be facilitated by on-farm testing. Monitoring and surveillance programs will benefit from a wide range of specimens that are simple, inexpensive and non-invasive to collect. Control projects will benefit from tests that improve our understanding of transmission and prevalence. Collectively, alternative diagnostic tests and specimens will provide timely, cost effective, and representative options for PRRS monitoring.

The validation methods, study quality, and amount of detail reported varied substantially for the various tests and specimens. Summary tables of the studies describing direct and indirect PRRSV tests are provided (Tables 5 & 6). These values are from independent studies which are not directly comparable. These tables are not intended to provide a consensus estimate of sensitivity or specificity. They have been compiled to provide a high level synopsis of the current published information. Readers are strongly encouraged to refer to each of the individual publications before using these values.

Several pen-side tests are being developed. The RT-LAMP technology, while promising, was not consistently sensitive (43 to 100%) across studies and has had little scrutiny of reliability and repeatability. Most studies were based on regionally-limited samples (Chen et al. 2010; Rovira et al. 2009). Once refined, validation using North American field samples is imperative because the foundation of 4 to 6 primers increases the concern that sensitivity and specificity could be regionally confounded. Antibody tests using the immunochromatographic strip test technology appear to be closer to field application. The BioSign™ PRRSV test has applied for USDA approval and a second test is being developed in Canada (AriVac Inc., Saint-Hyacinth, QC, Canada). Both tests have been developed and validated using pigs experimentally exposed to Type 2 PRRSV and clinical samples from North America. The BioSign™ product reported sensitivity and specificity >98%. This area of test development should be followed closely.

Several tests were described that provide promising options for laboratory-based diagnostics. Most notably, the potential to expand PRRS PCR testing to multiplex reactions for other viral pathogens, including FAD, is intriguing. Providing information on several diseases at once could offset PRRS monitoring costs and increase participation by producers with lower thresholds of concern for PRRS. If FAD were included, potential partnerships with government exist given the CFIA’s challenges in accessing representative and timely samples. Other direct-tests were also described including an antigen capture ELISA and an ICST based on antigen. As ARC&E projects achieve success and the goal becomes rapid time-to-detection and time-to-response to new infections, we foresee in increased reliance on direct tests. This could increase the market for such testing options.

Nine diagnostic specimens were described that could serve as alternatives to serum obtained by venipuncture from individual animals. Three had minimal detail reported and are not summarized (tonsil swabs, milk and neonatal tissues). RT-PCR testing on pooled serum, blood soaked swabs, and capillary tubes are in wide-spread use. Pooled serum samples should be encouraged for both
PCR and ELISA tests under certain circumstances. Veterinary diagnostic laboratories should be encouraged to offer this testing with optimized cut-off values. We support the immediate acceptance of such results for PRRSV control programs given the clear demonstration that the benefits to herd-sensitivity outweigh losses to individual sample sensitivity.

Oral fluids are also being rapidly adopted. The Iowa State University Veterinary Diagnostic Laboratory conducts up to 25% of PRRSV tests on oral fluids (Prickett et al. 2010a). When oral fluids are used for RT-PCR testing the viral concentration is lower, the time to detection is similar, and the window of detection is equivalent or slightly longer window than serum. The main advantage is the ease of sampling many pigs with a single test. This appears to result in an equivalent or improved probability of virus detection early in the course of infection (Prickett et al. 2010b; Prickett et al. 2011). Like all direct-test monitoring protocols, testing should occur every 2 to 3 weeks.

The collective research devoted to oral fluids samples provides confidence that this diagnostic specimen can provide precise, repeatable results. Reproducibility is the main remaining question about RT-PCR testing on oral fluids. This has been partly addressed by the reported optimized methods (Chittick et al. 2011), but Canadian laboratories may require assistance to adapt these procedures to their existing protocols. To date, there is insufficient evidence to support ELISA tests on oral fluids. Considering that Iowa State University Veterinary Diagnostic Laboratory is offering this test commercially and that researchers based out of this institution are responsible for over ¾ of the publications on oral fluids, we predict that addition information will soon be available that may change this position.

Filter papers offer several advantages as a specimen storage media. First, a range of specimens can be used including tissue and blood. For blood, the small volume required means that a minimally trained person can sample any age of pig without assistance. Despite a lower analytical sensitivity, the use of filter papers in pooled testing has been supported. Filter papers may also improve a monitoring system’s efficiency through non-quantifiable “human factors”. Long term storage at room temperature minimizes the potential for improper shipping and handling to adversely affect results, and filter papers could encourage participation if users prefer to collect samples for later testing. We recommend the anticipated publication by Abbott et al which will compare the sensitivity of three commercially available papers be closely considered. This will determine if recommendations about filter papers need to be brand specific.

Meat juice sampling may be the most practical and cost-effective way to monitor progress towards PRRS control and continued disease freedom in successful areas. This diagnostic specimen eliminates the biosecurity concerns caused by on-farm sampling, removes sample collection responsibility and labor from the producer, can be collected from an unbiased sampling frame, and has no animal welfare implications. Like the other diagnostic specimens based on ELISA tests, veterinary diagnostic laboratories will need to be encouraged to offer this test with an optimized cut-off to account for the lower analytical sensitivity. We fully support further investigation into optimizing the laboratory component and evaluate the logistical and legal issues of collecting samples from abattoirs.
Table 5. Summary of reported sensitivity and specificity estimates for direct PRRSV tests for described tests, specimens, collection methods, storage devices, and pool sizes. (NR: Not reported)

<table>
<thead>
<tr>
<th>Test</th>
<th>Specimen &amp; Collection or storage device</th>
<th>Pool size</th>
<th>% Sensitivity (Range)</th>
<th>% Specificity (Range)</th>
<th>Number of animals (N of samples)</th>
<th>Reference test</th>
<th>Number of publications (N Peer Reviewed)</th>
<th>References (Primary author and year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>Blood / Venipuncture</td>
<td>5 to 10</td>
<td>82 - 94</td>
<td>97 - 100</td>
<td>29 &amp; NR</td>
<td>qRT-PCR</td>
<td>2 (1)</td>
<td>(Polson 2010; Rovira 2007a)</td>
</tr>
<tr>
<td>PCR</td>
<td>Blood / Swabs</td>
<td>1</td>
<td>83 - 100 (3 to 15 dpi)</td>
<td>100</td>
<td>29 &amp; 30 (210)</td>
<td>qRT-PCR on serum</td>
<td>2 (2)</td>
<td>(Patterson 2007; Rovira 2007a)</td>
</tr>
<tr>
<td>PCR</td>
<td>Blood / Swabs</td>
<td>5</td>
<td>92 (relative)</td>
<td>100</td>
<td>29</td>
<td>qRT-PCR on individual swabs</td>
<td>1 (1)</td>
<td>(Rovira 2007a)</td>
</tr>
<tr>
<td>PCR</td>
<td>Blood / Capillary Tube</td>
<td>1</td>
<td>100</td>
<td>NR</td>
<td>30 (210)</td>
<td>qRT-PCR on serum</td>
<td>1 (1)</td>
<td>(Patterson 2007)</td>
</tr>
<tr>
<td>PCR</td>
<td>Blood / Filter discs</td>
<td>1</td>
<td>55 - 100</td>
<td>57 - 99.5</td>
<td>6 - 240</td>
<td>qRT-PCR on serum</td>
<td>5 (1)</td>
<td>(Abbott 2011; Linhares 2010a; Torremorell 2011; Yoon 2010)</td>
</tr>
<tr>
<td>PCR</td>
<td>Oral Fluid / Rope</td>
<td>~ 20+</td>
<td>17-88</td>
<td>100</td>
<td>48 (209)</td>
<td>qRT-PCR on serum</td>
<td>3 (2)</td>
<td>(Chittick 2011; Kittawornrat 2011; Prickett 2011; Prickett 2008b)</td>
</tr>
<tr>
<td>PCR</td>
<td>Oral Fluid / filter disc</td>
<td>~ 20+</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>qRT-PCR on serum</td>
<td>1(0)</td>
<td>(Linhares 2010a)</td>
</tr>
<tr>
<td>PCR</td>
<td>Thoracic fluid</td>
<td>1 to 8</td>
<td>93.6</td>
<td>96</td>
<td>NR</td>
<td>VI &amp; IHC</td>
<td>1(1)</td>
<td>(Benson 2002)</td>
</tr>
<tr>
<td>PCR</td>
<td>Meat juice</td>
<td>1</td>
<td>NR</td>
<td>NR</td>
<td>&gt;1000</td>
<td>NR</td>
<td>2 (2)</td>
<td>(Larochelle 1997; Magar 1995)</td>
</tr>
<tr>
<td>PCR</td>
<td>Tonsil / Swab</td>
<td>1</td>
<td>100</td>
<td>100</td>
<td>20 &amp; 180</td>
<td>PCR on serum</td>
<td>1 (0)</td>
<td>(Fangman 2007)</td>
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<tr>
<td>RT-LAMP</td>
<td>Blood / Venipuncture / PM Tissue</td>
<td>1</td>
<td>43 to 100</td>
<td>100</td>
<td>20 to 122</td>
<td>nRT-PCR &amp; qRT-PCR</td>
<td>4 (4)</td>
<td>(Chen 2010; Chen 2008; Qin 2009; Rovira 2009)</td>
</tr>
<tr>
<td>Ag Capture ELISA</td>
<td>Blood / Venipuncture / PM Tissue</td>
<td>1</td>
<td>67</td>
<td>97</td>
<td>466</td>
<td>qRT-PCR</td>
<td>1 (1)</td>
<td>(Cai 2009)</td>
</tr>
<tr>
<td>ICST</td>
<td>Blood / Serum / Tissue</td>
<td>1</td>
<td>97</td>
<td>94</td>
<td>25 &amp; 100</td>
<td>qRT-PCR</td>
<td>1 (1)</td>
<td>(Zhou 2009)</td>
</tr>
</tbody>
</table>
Table 6. Summary of reported sensitivity and specificity estimates for indirect PRRSV tests for described tests, specimens, collection methods, and storage devices, and pool sizes. (NR: Not reported)

<table>
<thead>
<tr>
<th>Test</th>
<th>Specimen &amp; Collection or storage device</th>
<th>Pool size</th>
<th>Optimized cut-off</th>
<th>Diagnostic Sensitivity % (Range)</th>
<th>Specificity % (Range)</th>
<th>Number of animals (samples)</th>
<th>Reference test</th>
<th>Number of publications (N Peer Reviewed)</th>
<th>References (Primary author and year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>Blood / Venipuncture</td>
<td>5</td>
<td>0.2 - 0.4</td>
<td>70 to 80</td>
<td>98 &amp; 100</td>
<td>113 &amp; 200</td>
<td>Individual serum with ELISA</td>
<td>2 (2)</td>
<td>(Hutet 2003; Rovira 2008)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Blood / Swabs</td>
<td>1</td>
<td>0.08</td>
<td>100</td>
<td>100</td>
<td>30 (210)</td>
<td>Individual serum with ELISA</td>
<td>1 (1)</td>
<td>(Patterson 2007)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Blood / Capillary Tube</td>
<td>1</td>
<td>0.4</td>
<td>93-100</td>
<td>100</td>
<td>30 (210)</td>
<td>Individual serum with ELISA</td>
<td>1 (1)</td>
<td>(Patterson 2007)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Blood / Filter discs</td>
<td>1</td>
<td>NR</td>
<td>86 to 100</td>
<td>100</td>
<td>6 to 200</td>
<td>Individual serum with ELISA</td>
<td>3 (2)</td>
<td>(Inoue 2007; Yoon 2010)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Blood / Filter discs</td>
<td>5</td>
<td>0.4</td>
<td>79</td>
<td>86 - 97.5</td>
<td>200</td>
<td>Individual filter discs with ELISA</td>
<td>1 (1)</td>
<td>(Hutet 2003)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Oral Fluid / Rope</td>
<td>~20+</td>
<td>NR</td>
<td>80</td>
<td>100</td>
<td>NR</td>
<td>Individual serum with ELISA</td>
<td>2 (0)</td>
<td>(Dufresne. 2011; Prickett 2010b)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Meat juice</td>
<td>1</td>
<td>0.1</td>
<td>&gt;96</td>
<td>100</td>
<td>91</td>
<td>Individual serum with ELISA</td>
<td>1 (1)</td>
<td>(Molina 2008)</td>
</tr>
<tr>
<td>ICST</td>
<td>Blood / Venipuncture</td>
<td>1</td>
<td>NR</td>
<td>96.7 - 98.7</td>
<td>97.8 - 99.3</td>
<td>nr, 650 &amp; 810</td>
<td>Individual serum with ELISA</td>
<td>1 (1)</td>
<td>(Achacha 2010; Cui 2008; Lyoo 2005)</td>
</tr>
<tr>
<td>gel-EIA</td>
<td>Blood / Filter discs</td>
<td>1</td>
<td>NR</td>
<td>93.7</td>
<td>100</td>
<td>150</td>
<td>Individual serum with ELISA</td>
<td>2 (0)</td>
<td>(Mende 2005)</td>
</tr>
</tbody>
</table>
CONCLUSIONS AND RECOMMENDATIONS

Test validation is laborious and costly for diagnostic laboratories (Greiner et al. 2000; World Organization for Animal Health (OEI). 2010a). For non-reportable diseases, there are guidelines but little standardization or external scrutiny of test validation (Christopher-Hennings et al. 1998). Dr. Christopher-Henning has described the validation steps for an in-house RT-PCR and concluded that “all of this information should be available to any practitioner or producer requesting the information”.

The reality is that veterinary diagnostic laboratories exist in a commercial environment and diagnostic test methods, including validation results, may be proprietary. Novel tests or techniques are unlikely to be offered without substantial field demand and when they are offered it is naïve to expect laboratories to share information material to their business. Given these realities, the Canadian swine industry should support a system that ensures tests are robustly validated without developing cumbersome or inefficient requirements that slow or prevent the development of novel tests and applications. To address this, we make three recommendations.

First, a ring-test study to compare PRRSV diagnostic test results on traditional samples and tests should be conducted. These results would establish the current performance, identify weaknesses, and determine the value of ongoing PRRSV proficiency panels for standard and alternative tests.

Secondly, funding for validation studies could expedite the technology transfer of new diagnostic tests to Canadian laboratories. Requirements of this funding should include an adherence to standardized validation approaches (World Organization for Animal Health (OEI). 2010b), publication in peer-reviewed journals with full disclosure of study design and reporting as outlined in QUADAS, and the use of samples that can validly be extrapolated to the Canadian population (O'Connor A.M. et al. 2007). Studies conducted internationally meet the needs of that particular region: Chinese studies typically focus on low-cost / low-equipment tests while American publications often validate automated procedures. Canadian laboratories are between these extremes and may require adaptations for our facilities, expertise or equipment.

Finally, laboratories could be encouraged to offer commercial testing on alternatives through access to samples for validation and proficiency testing. At least two institutions exist in the USA that provide samples for PRRSV proficiency panel testing (APHIS USDA, Diagnostic Virology Laboratory, National Veterinary Services Laboratories & Boehringer Ingelheim Vetmedica) but samples are costly and may or may not reflect Canadian isolates. Further, the proficiency panels offered are blinded so that Canadian veterinarians are not aware if their laboratory participates let alone their success rates.

There are precedents for each of these recommendations in Europe. A ring test has been conducted in Germany to evaluate the intra-laboratory agreement of PRRSV RT-PCR testing and raised concerning results (Truyen et al. 2006). In the French ‘Pays de Loire’ control area a study validated a cost-effective protocol for using pooled serum and filter discs to conduct PRRSV monitoring and decreased testing costs per herd by 75%. This same study subsequently supported laboratories by providing a calibrated standard to avoid variations in sensitivity between batches (Hutet et al. 2003).
The decision to offer new diagnostic tests will largely fall to the veterinary diagnostic laboratories in Canada. In contrast, producers and veterinarians will largely determine the uptake of pen-side diagnostic tests and alternative specimens. The main reason this review was undertaken was to evaluate if there is sufficient knowledge about these alternative tests and specimens to support in-field application. For individual farm decisions, we hope that this review has provided adequate detail to enable veterinarians to weigh the gained efficiencies against the remaining uncertainties. But for programs or regional health decisions the question remains, “Who decides what degree of uncertainty is acceptable?”

Our recommendation is that PRRS control programs join the international movement of accepting all forms of discrepant information that contribute to knowledge about diseases status (Cannon. 2002; Martin et al. 2007a; Martin et al. 2007b; OIE. 2010). Each observation and diagnostic result that provides any knowledge should be recognized and captured -- including results from alternative diagnostic tests or specimens. To fail to do so is a disservice to producers and forces undue costs on the industry. However, no alternative described in this review provided sufficient evidence to conclude that it is reproducible across laboratories and externally valid across the Canadian swine population. Therefore, we also recommend that these same programs institute quality control systems that validate a representative portion of samples against a reference test and specimen. As results are generated and confidence gained, the validation sample size can be continually updated in a Bayesian approach. We recognize that this recommendation will cost money and add logistical complications. However, the industry has experienced repercussions from inaccurate tests in the past and it is important to learn from these mistakes (Fetzer et al. 2006). Presuming this work is publically funded, results should be shared openly to instill interest, acceptance, and uptake of each alternative in the field.

Collectively, all of our recommendations could be addressed through a PRRS reference laboratory. The Canadian Swine Health Board might consider mediating discussions among veterinary diagnostic laboratories, researchers, and practitioners. While reference laboratory activities should not be limited to PRRSV, in the context of this report such an establishment could promote or coordinate PRRSV diagnostic test research in Canada. This could include validating tests using cost-effective pools or specimens, assist with the technology transfer of new techniques to existing laboratories, provide confirmatory testing for questionable results, offer proficiency panels, and possibly even offer certification as is currently done for reportable diseases.

For any activity, there is a limit to the human, time, and financial resources available (Cannon. 2009). Three “best” ways to allocate resources in monitoring have been described. These include maximizing the probability of disease detection, minimizing the time to detection, and maximizing the benefits arising from early disease detection. The first is a useful concept for point in time surveys while the latter two apply to ongoing monitoring efforts. For each, the best allocation is determined by comparing the efficiency per dollar spent. Voluntary endemic disease control is a novel initiative for the Canadian swine industry. Through this review, we conclude that many alternative diagnostic tests and specimens will improve the efficiency of these efforts. We support the appropriately cautioned use of these alternatives and wish to encourage users to evaluate diagnostic results for the information they provide about the population rather than the information they provide about the specimen.
Table 7. List of commercial products cited.

<table>
<thead>
<tr>
<th>Company</th>
<th>Product</th>
<th>Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>a Applied Biosystems</td>
<td>SYBR® Green TaqMan®</td>
<td>Streetsville, Ontario <a href="http://www.apppliedbiosystems.com">www.apppliedbiosystems.com</a></td>
</tr>
<tr>
<td>b AnVac Inc.</td>
<td></td>
<td>St. Hyacinthe, QC</td>
</tr>
<tr>
<td>c BD (Becton Dickson)</td>
<td>Vacutainer™</td>
<td>Franklin Lakes NJ <a href="http://www.bd.com">www.bd.com</a></td>
</tr>
<tr>
<td>d Biovet</td>
<td>Bio-Tubes</td>
<td>St-Hyacinthe, QC <a href="http://www.bio-vet.com">www.bio-vet.com</a></td>
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<tr>
<td>e Boehringer Ingelheim Vetmedica</td>
<td>Inglevac MLV</td>
<td><a href="http://www.bi-vetmedica.com">www.bi-vetmedica.com</a></td>
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<td>f IDEXX Laboratories</td>
<td>PRRS Ab Tests</td>
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<td>g Microvettes®</td>
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<td>RefWorks®</td>
<td><a href="http://www.refworks.com">www.refworks.com</a></td>
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<td>k RAM Scientific</td>
<td>Safe-T-Fill</td>
<td>Yonkers NY USA <a href="http://www.ramsci.com">www.ramsci.com</a></td>
</tr>
<tr>
<td>l Innovative Technologies for Life (ILT) Animal Healthcare</td>
<td>Tego ABC kits</td>
<td>Reston, VA <a href="http://www.iti-limited.com">www.iti-limited.com</a></td>
</tr>
</tbody>
</table>


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