DEVELOPMENT OF ELECTRONIC MICROARRAY ASSAYS FOR THE DETECTION OF HIGH CONSEQUENCE SWINE VIRUSES

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A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfilment of the
Requirements for the Degree

MASTER OF SCIENCE

Department of Biological Sciences
University of Lethbridge
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Date of Defense: March 27, 2019

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I would like to dedicate this thesis to my family. After all this time, they all believed in my abilities and supported me through the many trials and tribulations these last years. My parents, my siblings and most of all my loving wife; I thank you.

Abstract

This thesis describes the development and optimization of a seven-plex reverse transcription polymerase chain reaction (RT-PCR) and corresponding user-friendly electronic microarray for the detection of seven swine viruses: foot-and-mouth disease, swine vesicular disease, classical swine fever, vesicular exanthema of swine, African swine fever, porcine circovirus type 2 and porcine reproductive and respiratory syndrome. A panel of 58 strains of the viruses were successfully amplified and detected specifically on the NanoChip 400 microarray system while having no detection of 22 non-specific clinical material and non-target viruses and bacteria. Target viruses were also detected from clinical and biological materials spiked with viruses as early as 1 days post-infection. Detection limits ranged from 10 to 1000 copies for the targets. The assay was successfully transferred to the prototype Nexogen MDx portable and integrated system, where the fully automated system, with no manual handling from sample to detection, detected five of the seven viruses.

Acknowledgements

I want to acknowledge my friends and co-workers. My lab manager Tara Furukawa-Stoffer, for her knowledge and experience that helped guide me through this project and for her friendship when I hit my breaking point. My co-workers and friends: Kyle, Mat, Rodrigo and Cody, for all their help in the designing and running of my experiments as well as someone to bounce ideas and thoughts off of and share in the graduate school experience with.

My co-supervisor, Brent Selinger, I thank you for your support and help with my thesis writing and preparation. To Oliver Lung, for your guidance and tutelage since I began in your lab; you were the one that opened my eyes to the wonders of virology and research, as well as taught me valuable lessons about experimental design and writing, Thank you. To my committee members, James Thomas and Ed Taboada, thank you for taking the time to be on my committee.

My project was funded by the Chemical, Biological, Radiological-Nuclear and Explosives (CBRNE) Research and Technology Initiative (CRTI) and the Canadian Food Inspection Agency (CFIA) National Centre for Animal Diseases (NCAD) Lethbridge Laboratory.

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List of Abbreviations

Chapter	Full Description	Abreviation
1	African swine fever virus	ASFV
	Avian influenzia virus	AIV
	Bovine spungiform encephalophathy	BSE
	Canadian Food Inspection Agency	CFIA
	Classical swine fever virus	CSFV
	Ebola virus	EBOV
	enzyme-linked immunosorbent assay	ELISA
	Foot-and-mouth disease	FMD
	Foot-and-mouth disease virus	FMDV
	Gold Standard Tests	GST
	Hemagglutinin	HA
	Human immunodeficiency virus	HIV
	National Centre for Animal Disease	NCAD
	National Centre for Foreign Animal Disease	NCFAD
	Neuraminidase	NA
	Next generation sequencing	NGS
	Non-structural proteins	NSP
	Office International des Epizooties/ World Organization for Animal Health	OIE
	Point-of-care	POC
	Porcine epidemice diarrhea virus	PEDV
	real-time polymerase chain reaction	qPCR
	Reverse transcription PCR	RT-PCR
	Swine vesicular disease virus	SVDV
	United Kingdom	UK
	Vesicular exanthema of swine virus	VESV
	Virus Isolation	VI
	World Reference Laboratories	WRL
	World Trade Organization	WTO

2	5` Red specific reporter	5RS
	Adenosine triphosphate	ATP
	African swine fever virus	ASFV
	Base Pair	bp
	Basic local alignment search tool	BLAST
	Bio-containment level 3 Laboratory	BSL3
	Canadian Food Inspection Agency	CFIA
	Cap down A buffer	CdA
	classical swine fever virus	CSFV
	Cytidine triphosphate	CTP
	Deoxynucleic triphosphate	dNTP
	Dimethyl sulfoxide	DMSO
	Enzyme-linked immunosorbent assay	ELISA
	Escherichia coli	E.coli
	Ethidium Bromide	EtBr
	European	EU
	Foot-and-mouth disease	FMD
	foot-and-mouth disease virus	FMDV
	Foreign animal disease	FAD
	Guanosine triphosphate	GTP
	High Salt Buffer	HSB
	Integrated DNA Technologies	IDT
	Limit of Detection	LOD
	Lysogeny broth	LB
	National center for biotechnology informaiton	NCBI
	National Centre for Foreign Animal Diseases	NCFAD
	No template control	NTC
	Non-specific binding probe	NSBP
	North American	NA
	phosphotriester	PTE
	Point of Care	POC
	porcine circovirus type 2	PCV 2
	porcine reproductive and respiratory syndrome virus	PRRSV
	Positive:Negative	PN
	Red universal reporter	RUR
	Reverse biasing	RB
	Reverse transcription PCR	RT-PCR
	San Miguel sea lion virus	SMSV
	Sodium dodecyl sulphate	SDS

	Sodium Hydroxide	NaOH
	Super optimal broth with Catabolite repression	SOC
	SuperScript® III	SSIII
	Swine vesicular disease virus	SVDV
	United States	US
	Uridine triphosphate	UTP
	Vesicular exanthema of swine virus	VESV
	Virus Isolation	VI
	World organisation for animal health (Office International des Epizooties)	OIE
3	Adenosine triphosphate	ATP
	African swine fever virus	ASFV
	Base Pair	bp
	Canada Food Inspection Agency	CFIA
	Cap down A buffer	CdA
	classical swine fever virus	CSFV
	coefficient of variation	CV
	Cytidine triphosphate	CTP
	Deoxyribonucleic acid	DNA
	enzyme-linked immunosorbant assay	ELISA
	European	EU
	foot-and mouth disease virus	FMDV
	foot-and-mouth disease	FMD
	Foreign animal disease	FAD
	Guanosine triphosphate	GTP
	Low salt buffer	LSB
	Medical diagnostics	MDx
	National Centre for Animal Diseases	NCAD
	National Centre for Foreign Animal Diseases	NCFAD
	Non-specific binding probe	NSBP
	North American	NA
	Phosphate buffered solution	PBS
	Point of Care	POC
	Polymerase chain reaction	PCR
	porcine circovirus type 2	PCV 2
	porcine reproductive and respiratory syndrom virus	PRRSV
	Positive:Negative	PN
	Real-Time PCR	qPCR
	Red universal reporter	RUR
	Reverse transcription	RT
	Ribonucleic acid	RNA

	Sample buffer A	SBA
	Swine vesicular disease virus	SVDV
	Uridine triphosphate	UTP
	Vesicular exanthema of swine virus	VESV
	Wash buffer I	WBI
	Wash buffer II	WBII
	World organisation for animal health (Office International des Epizooties)	OIE
4	African swine fever viruse	ASFV
	Chemistry and Reagent Device	CARD
	classical swine fever	CSF
	Foot-and-mouth disease	FMD
	insolated isothermal PCR	iiPCR
	Medical diagnostics	MDx
	Point-of-care	POC
	Recombinase PCR amplification	RPA
	Reverse transcription PCR	RT-PCR

Chapter 1. Molecular Tools for Veterinary Diagnostics

1.1.0. Introduction

The risk of invasive species being introduced into naïve ecosystems has increased in recent years due to the rapid increase of global trading of goods. Invasive species can be any organisms that are not naturally found in another geographical range. The types of invasive species can range from animals and plants to microorganisms and viruses. The introduction of a foreign virus can be devastating and lead to an epidemic that costs agriculture industries billions of dollars. This has been demonstrated repeatedly in the past, including the outbreak of classical swine fever virus (CSFV) in the Netherlands (Terpstra and de Smit 2000), the 2003 occurrence of bovine spongiform encephalopathy (BSE) in Alberta Canada (Jones and Davidson 2014), the 2010 foot-and-mouth disease (FMD) outbreak in Korea (J.-H. Park et al. 2013) and the emergence of the porcine epidemic diarrhea virus (PEDV) in the United States in 2013 (Stevenson et al. 2013).

The World Organization for Animal Health, referred to as the OIE (Office International des Epizooties), is an international organization that deals with the improvement of animal health globally (http://www.oie.int/about-us/). The OIE acts as a hub of knowledge, creating an avenue for other countries to learn about possible diseases as well as the knowledge and expertise needed to protect against the pathogens causing these diseases. Their work is recognized by the World Trade Organization (WTO), allowing for standardized documents to be incorporated easily into other countries trade practices (http://www.oie.int/about-us/our-missions/). In 2018, 182 countries were listed

as members in the organization. These members create a network for the sharing of information and expertise as well as having international standards for animal welfare as well as trade standards with the WTO (http://www.oie.int/animal-welfare/oie-standards-and-international-trade/). These international standards are taken up by the member countries and administered by organizations such as the Canadian Food Inspection Agency (CFIA). Organizations such as the CFIA implement surveillance and diagnostic testing of the most harmful veterinary pathogens that can damage a country's economy by following the OIE standards. In 2001 the foot-and-mouth disease virus (FMDV) caused a massive outbreak in the United Kingdom (UK) resulting in approximately $13 billion USD in losses to producers, as well as 6.5 million animals being destroyed as a result of direct or indirect contact with infected farms (Thompson et al. 2002). Outbreaks such as this are the reason that world reference laboratories (WRL) such as the Pirbright Institute in the UK and the CFIA National Centre for Animal Diseases (NCAD) and National Centre for Foreign Animal Diseases (NCFAD) exist. The individual scientists at these WRLs, experts in their own fields, are who apply to the OIE to become a reference lab. These experts are required to be actively pursuing research in their field and are responsible for the assistance, both scientific and technical, to any other lab or member country that inquires assistance with their respective pathogen. Of the many responsibilities of WRLs, researchers must collect, store and distribute information and biological materials, as well as provide scientific and/or technical training for representatives from other member countries (http://www.oie.int/scientific-expertise/reference-laboratories/terms-of-reference/). The CFIA has multiple WRL experts that cover a range of diseases from anthrax to trichinellosis (Table 1). It is the

responsibility of individuals, such as Dr. Oliver Lung and Dr. Kingsley Amoako, to be a resource for advice and support in the surveillance, troubleshooting problems other groups may be having with their respective disease and report on their activities to the OIE.

1.2.0. Gold Standard Testing for High Consequence Pathogens

Viruses such as FMDV, CSFV, swine vesicular disease virus (SVDV), African swine fever virus (ASFV) and vesicular exanthema of swine virus (VESV) are all listed as federally reportable diseases by the OIE ("Federally Reportable Diseases for Terrestrial Animals in Canada - 2014" 2014) and can be considered high consequence pathogens due to their contagious nature and/or their indistinguishable symptomology. Any cases of these diseases found should be reported to the government and samples sent away for diagnostic confirmation at a licensed laboratory equipped to handle the appropriate risk group microoganisms and viruses. If during the diagnosis process any issues arise, such as failure to grow in culture or detection is weak or non-existent due to unknown serotypes, the corresponding WRL can be contacted for support and called upon to confirm any test results. One issue that can require additional confirmation is when symptomologies of two or more diseases are indistinguishable from one another. An example of this is the confounding clinical signs of FMDV and SVDV, both of which produce vesicular lesions on the foot and mouth of the animal (Fernández et al. 2008; Zimmerman et al. 2012, 603). By using laboratory testing, such as virus isolation (VI) and enzyme-linked immunosorbent assays (ELISA), technicians can determine whether

or not the pathogen is indeed present; helping to choose the best method for containment and management. Each of these diseases have multiple diagnostic tests that are outlined in “Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees)” which describes all the prescribed test methods used (OIE - World Organization for Animal Health 2018, http://www.oie.int/standard-setting/terrestrial-manual/access-online/).

The OIE can prescribe many different tests for pathogens from complement fixation, virus neutralization, VI, ELISA and real-time PCR (qPCR) for FMDV (OIE - World Organization for Animal Health 2013, chap. 2.1.8.). Though not all of the prescribed tests are often used, the ones that are most commonly used can be referred to as “Gold Standard Tests” (GST). Tests such as VI have GST status due to its high usage in diagnostic tests (D Deregt and Prins 1998; Ferris and Dawson 1988; Mittelholzer et al. 2006; Oka et al. 2014).

Though not all GSTs are validated according to OIE specifications, some are able to be validated into the OIE system after going through a rigorous testing process. The simplified development and validation pathway in Figure 1, referred from chapter 1.1.6 of the OIE diagnostic manual, defines the steps involved in validating a new diagnostic assay for use as an OIE GST. Any potential diagnostic test under evaluation must follow each category and meet each criterion. Gold standard tests are then implemented at appropriate WRL labs and other institutions where they can fulfill their mandate. The final purpose of OIE GSTs is to determine the absence of disease causing pathogens in geographical areas/countries, certify the health of animals bound for export/import, and

aid in eradication of the target pathogens (OIE - World Organization for Animal Health 2013, chap. 1.1.6.)

Gold standard tests are required to maintain a number of important characteristics. These characteristics include but are not limited to: Reproducibility, sensitivity, specificity and inclusivity. Repoducibility is required for all scientific work as it gives credibility to the project. If the work that is done cannot be reproduced in another laboratory setting, then the assay itself is called into question. A GST must be able to be transported from one diagnostic laboratory to another diagnostic laboratory, and be able to produce the same results. Selectivity and sensitivity refer to the ability of an assay to detect only the targeted organism and the lower limit of detection of the analyte, respectively (OIE - World Organization for Animal Health 2013; Thrusfield 2007). Most diagnostic tests are designed to detect and differentiate one target organism from related organisms or viruses. Each type of GST is designed to be specific, such as VI where each virus is grown in a particular medium and cell line. In cases such as the detection of FMDV, assays may have to be inclusive as well, being able to detect all possible serotypes of FMDV. FMDV has seven antigenic serotypes: A, O, C, Asia1, SAT 1, SAT 2 and SAT 3 that produce different surface antigens that can make them distinct in terms of how to vaccinate against one particular serotype (OIE - World Organization for Animal Health 2012, vol. 1, chap. 2.1.8). Inclusivity then can be an important tool in developing assays that detect not only multiple pathogens but multiple strains of organisms such as FMDV, SVDV and vesicular stomatitis virus (Lung et al. 2011).

The OIE GSTs have broad purposes. They help maintain statis quo in the economy, global trade and aid in epidemiological analysis'. Using the OIE standards, the

downstream users have readily available protocols, making trade and surveillance more streamlined. Methods such as VI, ELISA, qPCR and DNA microarrays are all useful tools for the detection of harmful pathogens.

1.3.0. Gold Standard Testing methods

1.3.1. Virus Isolation

Virus isolation is used as a standard protocol (Reid et al. 2003; Shaw et al. 2004) for virus research and diagnostics. The ability to infect a cell line with a known or unknown virus and maintain the growth in a cell line aids research by allowing scientists to work with viruses in a controlled environment. Emerging or transboundary pathogens challenge researchers to find and isolate the target pathogen in order to study it. In 2013, the PEDV was discovered in U.S. swine. PEDV is a contagious swine virus that causes watery diarrhea, dehydration, vomiting and has a 95% mortality rate in piglets (Stevenson et al. 2013; Chen et al. 2014). Using VI techniques, Qi Chen (2014) was successful in propagating the first example of the highly virulent PEDV strain (Oka et al. 2014).

1.3.2. Enzyme-linked Immunosorbant Assay

Alongside VI, ELISA is a commonly used GST. ELISA tests utilize immunochemistry to detect target antigens. Using antibodies, a body's immune-response to a foreign antigen, the user can detect target antigens by fixing antibodies to solid surfaces where the antigen can then bind and detected either directly or indirectly with

colorimetric substrates. The OIE include indirect capture ELISA as a recommended test for the detection and serotyping of FMDV (OIE - World Organization for Animal Health 2013, chaps. 2.1.8.; http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.08_FMD.pdf). Although using ELISA to detect the presence of FMDV in afflicted countries can also have a major drawback when utilized in areas that are currently FMDV free due to vaccine usage. Animals that have been infected can be almost indistinguishable from those vaccinated when being tested by ELISA. Vaccination of FMDV can inadvertently lead to the animal becoming persistently infected, which also leads to un-diagnosable ELISA results compared to infections (Mackay et al. 1998). To combat this, researchers found that in the case of a vaccinated versus non-vaccinated inoculations, the virus induced antibodies to the non-structural proteins (NSP) in its host and the NSP can be used to detect the infection status in a herd; whether or not the herd is clean, infected or vaccinated with or without a persistent infection (Mackay et al. 1998). Since this discovery, many groups have developed assays for the detection and differentiation of FMDV infected and vaccinated animals (Brocchi et al. 2006; Jaworski et al. 2011; Sørensen et al. 1998), in the effort to eradicate FMDV and create a disease free status for the afflicted country.

1.3.3. Nucleic Acid Tests

With the surveillance of invasive pathogens, the titre of the pathogen found inside the host organism can be too low to cause visual symptoms, allowing an infected host into a naïve ecosystem. More sensitive tests are needed to mitigate these situations.

Nucleic acid tests are a more sensitive alternative to serology tests such as ELISA (Mukasa et al. 2016; Reid et al. 2003; Shaw et al. 2004). PCR is capable of amplifying a target gene sequence billions of times, making even low quantity analytes detectable. With the introduction of fluorescent probes, instruments are now able to read the PCR amplification of a sample in real time and are able to calculate the quantity of target genetic material used in the sample. Real-time PCR has been used in detection of different pathogens from plant fungi (Vandemark et al. 2002; Willsey et al. 2018) to cattle viruses (Baxi et al. 2006; Carrillo et al. 2010; Hole et al 2006; Oem et al. 2005). Currently, qPCR has been used as the GST for the detection of FMD due to its ability to accurately detect low copies of viral genomes (Shaw et al. 2007; Oem et al. 2005; King et al. 2006). In cases such as the work from Galvin et al., (2014) qPCR was seen to detect equine influenza a full day post infection (dpi) earlier than ELISA, as well as able to detect 100% of positive samples while VI and ELISA detected only 69 and 2% respectively from post-experimentally infected horses.

1.3.4. Microarray

Besides qPCR, microarrays are another molecular technique that offer a more robust approach to detection using fluorophores. DNA microarrays are platforms that use oligonucleotide “capture probes” that, when fixed to a solid substrate surface, can anneal to nucleotide sequence specific “targets”. Microarray systems have the advantage of being able to use hundreds if not thousands of capture probes, each bound to specific testing sites on the substrate, that are used to detect multiple targets simultaneously

(Chang et al. 2012; Ojha and Kostrzynska 2008). Slide microarrays can contain thousands of probes and can be replicated multiple times on the substrate surface. Cases such as the detection of vesicular disease viruses, with the aid of multiplex reverse transcription PCR (RT-PCR), were able to not only detect the individual target viruses, but also to differentiate between the subtypes of both FMDV and other viruses that cause vesicular diseases (Lung et al. 2011). Electronic microarrays use an electric current to speed up hybridization. Given the negative charge on nucleic acids, using an electric current pulls the DNA towards the positively charged pads containing the capture probes. This gives the technique a unique advantage by reducing the time needed for hybridization from at least 16 hours (Wang and Li 2011) to a few seconds (Lung et al. 2012; Erickson et al. 2017). Microarray technologies have a distinct advantage in their detection ability. By utilizing multiple capture probes, inclusive assays can be developed. Using an example from Lung et al., (2012) Avian influenzia virus (AIV) can be detected and subtyped down to the specific hemagglutinin (HA) and neuraminidase (NA) subtype. The number of non-specific cross reactions between the different isolates was seen as specific enough that only one cross-reactivity between HA3 and NA8 probes was observed (Lung et al. 2012). An assay such as this makes it possible to detect dual infections of two or more isolates of the AIV as well as other pathogens (Lung et al. 2017).

Though VI, ELISA and qPCR are commonly used, they all have their disadvantages. Time, cost, infrastructure and technical support are limitations of each test. VI is a time consuming process that requires specialized lab spaces, equipment and skilled personnel to achieve results. Traditional microarrays can also be time consuming

as well as requiring a skilled technician to perform steps such as the fluorescent labeling of the DNA target for visualization after hybridization to the capture probes (Baxi et al. 2006; Lung et al. 2011).

1.4.0. Automated Diagnostic Methods

In the persuit of more rapid results, automation of assays have gained popularity. Examples of some automated systems are point-of-care (POC, or sample-to-answer) systems. Point-of-Care systems combine multiple components of the diagnosis process, for example, sample extraction, reverse transcription-PCR (RT-PCR) and detection, into one instrument. This cuts down greatly on the need for manual manipulation of samples and test materials, which reduces user error and variability, and chances of contamination of the sample leading to the generation of false positives. These systems can have a great range of operation, including partially automated and fully automated assays. Partially integrated assays use two of the three steps described above in combination: Front-end (extraction and PCR) and back-end (PCR and detection). In both cases, one step needs to happen in-lab, in the case of Jeslin Tan's work on tropical disease, the pathogens were extracted in-lab and then run on the PCR/RT-PCR Lab-on-Chip microarray system (Tan et al. 2014). The advantage of the Lab-on-Chip system is its ability to incorporate multiple targets into one assay, giving a thorough confirmation to the causative agent. Many different types of POC have been developed, ranging from back end PCR/detection assays (Teo et al. 2011) to fully integrated systems like the EncompassMDx™ workstation (Lung et al. 2018; Spizz et al. 2012, 2015). In the recent work of Chen et al. (2016), they described the use of the Encompass MdX SOLO instrument to detect antibodies and viral RNA of the human immunodeficiency virus (HIV) from saliva and

blood samples. Testing showed that the automated MdX SOLO was able to detect viral titres out to $2.5x10^3$ viral particles / mL with the on-board real-time system as well as being able to determine an extraction buffer that would allow for DNA extraction while not harming the protein antibodies for the secondary extraction of proteins (Chen and Zhu 2016). Instruments such as this are the future for medical care, allowing either hospital or veterinarian clinics to analyze patient samples on-site in an attempt to reduce the time needed for an accurate diagnosis. Development of new POC instruments is ongoing with many companies, such as Nexogen Inc. (San Diego, USA), SavyonDiagnostics (Ashdod, Israel), AdorDiagnostics (Rome, Italy), Abaxis (Union city, USA) and Roche Diagnostics (Risch-Rotkreuz, Switzerland). These companies, and many others, are working to create tests that incorporate all aspects of the diagnostic process to create instruments that save both time and labour.

1.4.1. Next Generation Sequencing

NGS is a powerful technique for genome sequencing. Wright et al (2011) used NGS to delve deeply into the microevolution of the FMD viral genome in an attempt to understand rapid viral RNA evolution (Wright et al. 2011). Emerging viruses such as PEDV are more troublesome due to the inability to propagate using VI techniques. During the 2013 outbreak in the U.S., PEDV was identified and propagated for the first time, allowing scientists to study this infectious pathogen. NGS was used as a confirmatory tool to identify the strain of PEDV that was involved in the outbreak (Stevenson et al. 2013; Chen et al. 2014). Work such as this has a goal of detecting not

only the target pathogen, but also other more opportunistic secondary pathogens. Thorburn et al. (2015) used NGS to identify multiple pathogens from upper respiratory infections of human patients from Influenza A HA3NA2 to Human coronavirus (Thorburn et al. 2015).

Further advancements in the field of nucleic acid sequencing have also given rise to a third generation of NGS instruments that are capable of increasing throughput by sequencing single molecules as well as giving the results in real time (Granberg et al. 2016; Greninger et al. 2015; Quick et al. 2016). One particular instrument, the MinION (Oxford Nanopore Technologies, Oxford, UK), utilizes a protein nanopore where single stranded DNA is passed through at 30 bases per second using an electrical current. In this instrucment, different nucleotides are detected by changes in ionic flow (Butt et al. 2018; Quick et al. 2016). Another strength of the Nanopore system is long read lengths, from 5 to 50 kb, being generated and the speed at which data can be acquired in real time (Butt et al. 2018; Greninger et al. 2015; Quick et al. 2016). During the 2015 Ebola virus (EBOV) outbreak in West Africa, a mobile sequencing laboratory was set up to process samples received for EBOV testing. Testing showed that results could be generated in under an hour with no false positives found (Quick et al. 2016). The longer reads of the MinION, while being critical for specific identification of targets, is hindered by a high error rate (between 5 & 30%). This is mitigated by running multiple replicate sequences and creating a consensus from a pairwise alignment, increasing the accuracy to as high as 90% (Butt et al. 2018).

1.5.0. Thesis Objectives

Though many of these tests focus primarily on detection, they give a foundation for other tests that can be useful in controlling outbreaks. The use of GSTs continues to improve by either increasing the sensitivity of a particular test, using multiple tests in combination to account for weaknesses in each test or by incorporating new techniques that either reduce the work load and/or increase the speed of detection using integrated platforms or vastly increase the level of detection by sequencing the samples, showing distinct strains and serotypes. The constant improvement of GST, in support of food and animal disease surveillance, will aid in the growth of the Canadian economy by reducing the number of major outbreaks that occur or by helping to mitigate outbreaks that may negatively impact the economy.

The work done in this thesis represents a step towards reducing work load and the integratation of platforms. There are two main objectives for this thesis. One, is the development of a multiplex RT-PCR assay on a user-friendly electronic microarray which will demonstrate the usefulness of automating the back-end of the diagnostic process (Chapter 2). It also illustrates the advantages of multiplexing multiple pathogen detection tests into one assay, creating a test to simultaneously detect a number of pathogens at once. The secondary objective will be to transfer the multiplex assay onto a fully integrated system, combining all major steps in the diagnosis process from extraction to detection. The integrated system is a collaborative effort with developers that wanted to create a test to fit their POC instrument (Chapter 3). This work adds to the knowledge base for pathogen detection by giving an account of the work done using my

selected pathogens and will promote future work in the field of veterinary diagnostics and POC assay development.

1.6.0. Figure Legend:

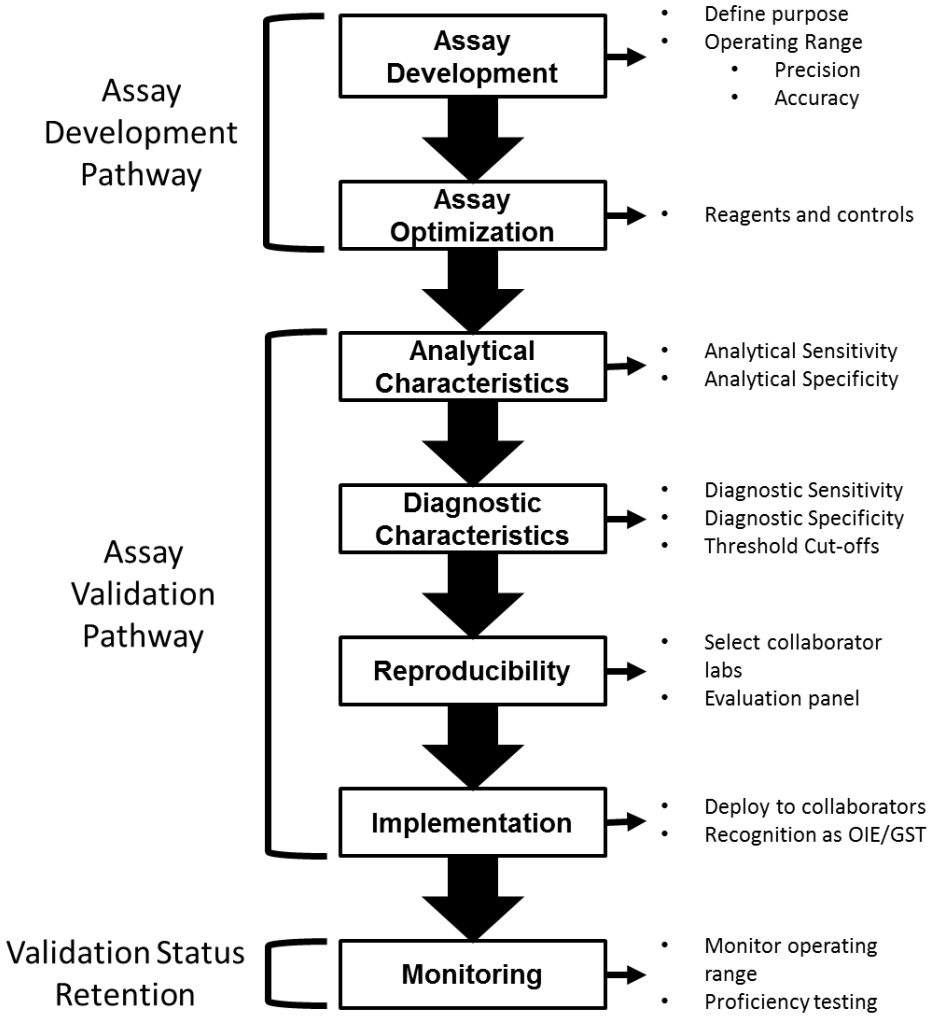

Figure 1.1. OIE assay validation pathway including criteria in bullet form (OIE – World Organization for Animal Health 2012, vol. 1, Chp. 1.1.6).The main pathway is broken down into three phases: Assay development, validation and status retention. Respective goals and objective are listed to the right, outlining at each step what is expected of developers.

1.6.1. Table Legend:

Table 1. List of current WRL applicants within the CFIA organization*

Disease	Researcher	Institute	Location
Anthrax	Dr Kingsley Amoako	National Centre for Animal Disease (NCAD)	Lethbridge, AB
Bovine spongiform encephalopathy	Dr Stefanie Czub	National Centre for Animal Disease (NCAD)	Lethbridge, AB
Bovine Viral Diarrhoea	Dr Oliver Lung	National Centre for Animal Disease (NCAD)	Lethbridge, AB
Classical swine fever	Dr Aruna Ambagala	National Centre for Foreign Animal Disease (NCFAD)	Winnipeg, MB
Avian influenza	Dr Yohannes Berhane	National Centre for Foreign Animal Disease (NCFAD)	Winnipeg, MB
Chronic wasting disease	Dr Gordon Mitchell	Animal Disease Research Institute (Fallowfield)	Ottawa, ON
Scrapie	Dr Gordon Mitchell	Animal Disease Research Institute (Fallowfield)	Ottawa, ON
Trichinellosis	Dr Brad Scandrett	Centre for Food-borne and Animal Parasitology	Saskatoon, SK

* http://www.oie.int/scientific-expertise/reference-laboratories/list-of-laboratories/

1.7. References

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Chapter 2. Development and Optimization of a User-Friendly Electronic Microarray Detecting Seven Swine Viruses[1]

2.1.0. Introduction

The red meat industry (eg. cattle, lamb, pork) is one of the largest industries in Canada, averaging approximately $6.1 billion annual domestic exports in 2017 (Statistics Canada 2018). Pork accounts for approximately 25% of the whole production of red meat totalling $4.1 billion in sales, and about 70% of all processed meats in Canada are made of pork (Negrave 2014). In 2011, Canada ranked as the fifth largest pork exporter (Brisson 2014), and in 2017 Canadian pork exports peaked at $4.2 billion (Statistics Canada 2018).

Animal health is an important issue affecting the livestock industry. Disease outbreaks may cause huge economic losses due to reduced growth and general health that makes the pigs unmarketable, have lower market value, much higher pharmaceutical costs and increased mortality. In 2005, the United States (US) attributed $561 million in loss caused by diseases such as porcine reproductive and respiratory syndrome virus (PRRSV) (Holtkamp et al. 2013), while in 2001 in the UK, an outbreak of foot-and-mouth disease (FMD) was responsible for losses upwards of $13 billion US (Thompson

[1] Pulished in: "Erickson, A., M. Fisher, T. Furukawa-Stoffer, A. Ambagala, D. Hodko, J. Pasick, D. P. King, C. Nfon, R. Ortega Polo, and O. Lung. 2017. "A Multiplex Reverse Transcription PCR and Automated Electronic Microarray Assay for Detection and Differentiation of Seven Viruses Affecting Swine." *Transboundary and Emerging Diseases*, November. https://doi.org/10.1111/tbed.12749." Anthony Erickson was the lead researcher in the project, conceptualizing and running all the experiments, as well as supporting work done in the Winnipeg laboratory.

et al. 2002). Losses such as these are made worse by reductions and embargos on trade with other countries during and after outbreaks. Early detection is thus important in mitigating potentially devastating diseases from decimating our livestock populations and harming our economy.

Foreign animal diseases (FAD) pose a great risk to economic stability as well as native or indigenous species (Thompson et al. 2002; Dirk Deregt et al. 2006) and also affects food security. FAD viruses can be highly contagious and may be easily transmitted through livestock populations, making surveillance of such diseases imperative. Many pathogens, regardless of genus or family, can cause diseases that cannot be readily distinguished by clinical signs and require laboratory testing for an accurate diagnosis. The viruses described here all have the potential for confounding initial diagnosis due to similar and/or identical clinical signs (Table 1). This study focuses on five high consequence viruses that are not currently observed in Canada and two differential viruses observed in Canada.

Foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family, is the most contagious animal virus, causing vesicular lesions in the mouth and hoofs of most cloven-hoofed animals including: cattle, horses, swine, sheep and all wild ruminants. (Lung et al. 2011; Zimmerman et al. 2012, 590; Fernández et al. 2008; Knipe et al. 2007). Swine vesicular disease virus (SVDV), also a member of the *Picornaviridae* family, is similar to FMDV where affected pigs also generate vesicular lesions (Zimmerman et al. 2012, 603; Knipe et al. 2007). Vesicular exanthema of swine virus (VESV), was a virus derived from feeding pigs seal meat contaminated with the San Miguel sea lion virus (SMSV) (Zimmerman et al. 2012, 493–94) from the *Caliciviridae*

family. Though VESV is considered an extinct virus, it is included in analysis because it can cause clinical signs that closely resemble clinical FMDV and SVDV cases (Zimmerman et al. 2012, 494).

Another highly contagious virus is the classical swine fever virus (CSFV). Belonging to the *Flaviviridae* family, CSFV causes hemorrhages in pigs (Giammarioli et al. 2008) and lesions in less severe cases (Zimmerman et al. 2012, 541–42). The African swine fever virus (ASFV), the only member of the *Asfarviridae* family, can be misdiagnosed as a CSFV infection due to similar hemorrhagic clinical signs (Costard et al. 2009; Giammarioli et al. 2008).

All five of the FAD viruses are responsible for significant economic disturbances throughout the globe. A CSFV outbreak in the Netherlands in 1997-1998 (Terpstra and de Smit 2000), the FMDV outbreaks in; the UK in 2001 (Thompson et al. 2002), Japan in 2010 (Muroga et al. 2012) and multiple outbreaks in South Korea from 2000-2011 (Joo et al. 2002; J.-H. Park et al. 2013) are but a few of the examples of past outbreaks. Each one of these outbreak events caused great economic losses ranging from $550 million to 13 billion. These viruses are all considered federally reportable diseases in Canada and by the World Organization for Animal Health, referred to as the OIE (International Office of Epizootics, “Federally Reportable Diseases for Terrestrial Animals in Canada - 2014” 2014). Their status is due to their potential impact on global trade, making these FADs a high priority for food security and surveillance.

Also included for analysis in this study are two viruses indigenous to North America; porcine circovirus type 2 (PCV 2) and PRRSV. Members of the *Circoviridae* and *Arteriviridae* families, respectively, they are responsible for most of the production

losses to the North American swine industry (Nicholson et al. 2011; Zimmerman et al. 2012, 405, 461 repectivly). Due to the etiology of these pathogens, the industry controls these viruses through rigorous sanitation and quarantine methods. These two pathogens are the causative agents for reproductive failure (Liu et al. 2013), lesions (Zimmerman et al. 2012, PCV2 410, PRRSV 470) and other clinical signs.

Tests such as enzyme-linked immunosorbent assays (ELISA), virus isolation (VI) and virus neutralisation are regarded as the gold standard tests for most of the above target viruses (Table 2.1) (OIE - World Organization for Animal Health 2018, chaps. 2.1.8, 2.8.1, 2.8.3, 2.8.6, 2.8.8,). Virus isolation typically requires at least 4-6 days to allow the virus to grow in cell cultures (Yamazaki et al. 2013). Recent advances in molecular biology have shown the use of reverse transcription polymerase chain reaction (RT-PCR) as a viable alternative for rapid and accurate detection of the viral targets. Single-plex RT-PCR assays use a single set of primers that target a specific genomic region of a particular virus or a group of genetically similar viruses. The power of a single-plex RT-PCR is the ability to detect low quantities of target RNA in a sample, as well as its ability to be specific to a particular genetic target (Desingu et al. 2015; S. M. Reid et al. 1999, 2000). Assays such as these have been used for detection and serotyping of FMDV (M. K. Baxi et al. 2006) and CSFV (Paton et al. 2000). One-pathogen/one-test can be a very efficient system when dealing with a single viral infection; however, in cases where multiple pathogens are suspected, the one-pathogen/one-test method requires the use of multiple tests being run against a single sample (Jiang et al. 2011; Hidalgo Ashrafi, Yee, and Paul 2009; Giammarioli et al. 2008). The increased number of tests needed for each sample increases sample, reagent and labour cost and time. An assay that

can simultaneously detect multiple genetic targets in a single reaction can improve the efficiency of diagnostic testing.

Multiplexing refers to the amalgamation of multiple sets of PCR primers for different pathogens into a single assay (Belák 2007; Liu et al. 2013; Lung et al. 2011). Multiplexing has the potential to enhance diagnostic testing by reducing costs and labour. In cases of co-infection involving multiple pathogens, a multiplex PCR test has the potential to simultaneously amplify and differentiate possible targets (Diaz de Arce et al. 2009; Ogawa et al. 2009; Lung et al. 2011, 2016, 2017).

Microarrays are detection platforms that utilize sequence specific "capture" probes that hybridize with single stranded complimentary DNA "target" amplicons (Miller and Tang 2009) and are visualized using either colormetric (Fici et al. 2010; Spizz et al. 2015) or fluorescent (Takahashi et al. 2008; Lung et al. 2012) reporters. Microarrays have the ability to interrogate the sample with a large number of capture probes simultaneously, allowing for a great degree of multiplexing potential. Originally, microarrays were used for gene expression experiments utilizing a large number of probes to detect the expression of hundreds of genes simultaneously in systems including porcine (Gao et al. 2012) and bovine (Wilson et al. 2005). While most microarrays use passive hybridization, amplicons diffusing passively through a medium towards the fixed probes; electronic microarrays use electrical currents to actively pull negatively charged amplicons towards the probes. The use of the electrophoretic migration greatly reduces the time needed for hybridization (Syrzycka et al. 2003) and allows for rapid generation of results. The NanoChip 400 (Nexogen Inc., San Diego, US) takes this a step further by

using 400 individual test sites that can each be independently activated and tested separately. This allows for a greater degree of flexibility in assay design and execution.

This study covers the development, optimization and validation of a seven-plex RT-PCR and electronic DNA microarray for the detection of five viruses exotic to Canada and two differential indigenous viruses that affect swine. The goal of this study is to develop an user-friendly assay that simplifies the diagnostic work flow for multi-pathogen detection, reducing both time and cost for diagnostic tests and decrease response times to outbreak situations.

2.2.0. Methods and Materials:

2.2.1.0. Samples

Seven high consequence viruses were selected for this study (Table 2.2). A panel of eight viral strains was used for initial testing of the seven-plex assay: FMDV SAT 3 BEC 1/65, SVDV ITL 19/99, CSFV Alfort187, ASFV Lisbon 61, VESV Cal, PCV2 B, PRRSV YNL and PRRSV LV. Two lineages of PRRSV, North American (NA) and European (EU), were included to validate the ability of the assay to subtype and differentiate the two virus lineages. Initial development was done with 1/50 dilutions of the neat extracted virus total nucleic acid material. Due to the biocontainment requirements for experimental infections and the exotic viruses, all work involving unextracted viruses were completed in the biosafety level 3 laboratory (BSL3) at the Canadian Food Inspection Agency (CFIA) National Centre for Foreign Animal Disease (NCFAD) in Winnipeg Manitoba.

Synthetic constructs of FMDV were made for the 981 base pair (bp) region encoding VP3/VP1/2A/2B portions of the polyprotein and used as a surrogate of viral RNA during assay development in Lethbridge due to CFIA policy that FMDV viral RNA can only be used in the Winnipeg BSL 3 laboratories. RNA transcribed *in vitro* from plasmids containing synthesized DNA of FMDV, SVDV, CSFV, VESV and PRRSV, as well as plasmids, containing sequences of the two DNA viruses PCV 2 and ASFV, were used to determine the analytical sensitivity of the assay. RNA *in vitro* transcribed from amplicons of FMDV sequences was also used for development of the multiplex assay.

Clinical material for 17 strains of four target viruses; FMDV, SVDV, CSFV and ASFV was obtained from experimentally inoculated pigs infected at the CFIA NCFAD laboratory. A number of oral and nasal swabs, whole blood and serum samples were collected and a final panel of samples were selected for testing (n=114). A total of 100 oral and nasal swabs taken from <2 day old piglets (n=15) and >2 day old hogs (n=10), were obtained from Prairie Swine Centre, Saskatoon, Saskatchewan to be used as negative clinical material for specificity testing and virus spiking.

2.2.1.1. Cloning and *in vitro*-Transcription of Target Viruses

The target genes of SVDV, VESV, ASFV, PCV2 and PRRSV (Table 2.2) were amplified by RT-PCR reaction using Invitrogen's SuperScript® III OneStep RT-PCR with Platinum® Taq kit (Life Technologies - Invitrogen, Burlington, ON). A 50 µL PCR reaction was made using the following reagents: 0.5-1 µL of the specific primers (50µM) for each individual target, 25 µL of 2x RT buffer mix, 2 µL of SSIII enzyme mix, 20-21

µL of ultra-pure water and 1 µL of target sample. The reactions were run on the Veriti 96 well thermal cycler (Life Technologies-Applied Biosystems, Burlington, ON) at the following conditions: 55 °C RT for 15 min then 94 °C initial denaturation for 2 min, followed by 35 cycles of: 94 °C denaturation for 30 s, 50 °C annealing for 1 min and 68 °C extension for 1 min and a final 68 °C extension for 5 min. All post-amplification RT-PCR amplicons were visualized on the QIAxcel instrument (Qiagen, Toronto, ON). Amplicons were desalted and purified using the ZYMO DNA Clean & Concentrator™-5 column kit (Zymo Research Corp., Irvine, CA, USA) and quantified on the NanoDrop 8000 UV-Vis Spectrophotometer (ThermoScientific-NanoDrop, Wilmington, DE). Quantified amplicons for the five targets were treated with DNA blunting enzyme from the CloneJET PCR cloning kit (ThermoScientific-Fermentas, Ottawa, ON) for 5 min at 22 °C. The amplicons were ligated to 1 µL of pJET1.2 (50 ng/µL) vector (ThermoScientific-Fermentas, Ottawa, ON) in a 3:1 insert:vector ratio, with 1 µL T4 ligase (1U), at room temperature for 30 min.

Ligated material was transformed into chemically competent "One Shot® MAX Efficiency® DH5α™-T1^{R}" *Escherichia coli* (*E.coli*) cells. On ice, 3 µL of ligated material was incubated with 20 µL of cells for 30 min. The reaction was then heat shocked at 42 °C for 30 s, cooled on ice and inoculated with 250 µL of prewarmed 37 °C SOC media, and stored for 1 hour at 37 °C. 100 µL of cells were then plated on a LB Miller agar plate containing 100 µg/µL Ampicillin (100 mg/mL) and incubated at 37 °C overnight. Colonies were counted and a colony PCR was performed using 0.3 µL Taq polymerase (MP Biomedicals, Santa Ana, CA), 2 µL 10x Taq buffer, 0.4 µL deoxynucleotide triphosphate (dNTP, 10mM), and 0.4 µL of each of the pJET1.2 forward

and reverse primers. PCR reactions were run at 95°C for 3 min followed by 30 cycles of: 93 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min with a final extension step for 10 min at 72 °C. Five mL of overnight liquid cultures of selected colonies were set up in LB broth containing 5 µL of 100 µg/µL Ampicillin and incubated at 37 °C overnight. One mL glycerol stocks were made at 1:1 ratios with 60 % glycerol and stored at -80 °C. The remaining culture was pelleted at 14000 rpm for 3 min. The concentrated cells were extracted using the Qiagen QIAprep miniprep plasmid extraction kit (Qiagen, Toronto, ON) and the resulting pDNA was quantified on the NanoDrop 8000.

A Zero Blunt® TOPO® PCR cloning kit was used (Life Technologies - Invitrogen, Burlington, ON) to clone the CSFV E1/E2 gene. Briefly, the E1/E2 gene of CSFV was amplified with Platinum® Taq DNA polymerase High Fidelity (Life Technologies - Invitrogen, Burlington, ON) in a 50 µL PCR reaction with the following reagents: 1 µL of the CSFV primers (50 µM, Table 2.3), 5 µL of 10x high fidelity buffer, 1 µL of dNTPs (10 mM), 50mM $MgSO_4$, 0.2 µL of Platinum® Taq polymerase (5 U/µL), up to 50 µL of ultra-pure water and 1µL of template RNA. The reactions were run on the Veriti thermocycler at the following conditions: 94 °C for 2 min, followed by 35 cycles of: 94 °C for 30 s, 50 °C for 1 min and 68 °C for 1 min and followed by a final extension of 68 °C for 5 min. After analysis on QIAxcel, the products were purified and ligated in a TOPO® blunt ligation reaction with 0.25 µL salt solution, 0.38 µL blunt amplicon, 0.25 pCR™II-blunt-TOPO® vector and up to 1.5 µL H2O. The reaction was incubated for 30 min at room temperature (~22 °C) and then chemically transformed into TOP10 chemically competent *E.coli* cells following the same procedure as the pJET1.2

transformation with the exception that transformants were plated on LB agar plates and then grown in broth containing 100 µg/mL Kanamycin.

FMDV synthetic constructs, containing the desired genomic regions, were synthesized by Integrated DNA Technologies (IDT) in the pGEM-3Zf(+) vector. FMDV plasmids were transformed into DH10B *E.coli* electro-competent cells. Briefly, 1 µL of plasmid was added to 20 µL electro-competent cells and incubated on ice for 5 min. The 21 µL mixture is then added to a 1mm cuvette and electroporated in the BioRad Gene Pulser Xcell™ electroporator unit (Bio Rad, Mississauga, ON) at: 1800 volts, 25 capacitance and 200 resistance. After electroporation, 500µL of prewarmed SOC media is added and the cells were incubated at 37 °C for 1 hr. After incubation, the culture was plated as described above for CSFV.

Quantified plasmid for SVDV, CSFV, VESV and PRRSV were restriction digested in a 250 µL reaction consisting of approximately 5µg plasmid DNA, 25 µL 10 Rx buffer, 5 µL HindIII (10u/µL) (Thermo Scientific-Fermentas, Ottawa, ON), and up to 250 µL dH2O. Plasmids in a volume of 25 µL were also run in a undigested control without the restriction enzyme and with 1/10 the volume of the template used in reactions with enzyme. Reactions were incubated at 37 °C for 16 hours. Digests were confirmed by running a 1 % UltraPure™ Agarose (Life Technologies-Invitrogen, Burlington, ON) gel at 100 volts for 45 min. The plasmid for FMDV was digested similarly but with 10 µL EcoRI enzyme (10U/µL) and 50 µL 10x Tango buffer (Life Technologies- Thermo Scientific-Fermentas, Ottawa, ON). Digestions were terminated by adding 12.5 µL 0.5M EDTA, 25 µL 3M sodium acetate and 500 µL ethanol (EtOH) to the reaction, incubated at -20 °C for 20 min and then centrifuged at 14000 rpm for 15 min at 4 °C. The

supernatant was decanted and the pellet was resuspended with 10 µL 1x TE buffer pH 8 (Integrated DNA Technologies, Coraville, Iowa). The resuspended material was then treated with 1 µL of 2 µg/mL protease K and 0.5 µL 10 % Sodium Dodecyl Sulfate (SDS) and incubated at 50 °C for 30 min. Following treatment, material was purified using the Zymo column kit and eluted with 0.1xTE pH 8.0 and then quantified by NanoDrop.

For RNA *in vitro* transcription, the MEGAscript® kit (Life Technologies – Invitrogen, Burlington, ON) was used for all RNA viruses (SVDV, CSFV, VESV, PRRSV) except for FMDV. A reaction mix was made up for the *in vitro* transcription containing; 1 µg of plasmid, 2 µL of each 75 mM dNTP (ATP, GTP, CTP and UTP), 2 µL of 10x reaction buffer, 2 µL enzyme mix and up to 7 µL nuclease-free water for a final volume of 20 µL. The *in vitro* transcription reaction was continued for 4 hours at 37 °C. Template DNA was removed by digestion with 3 µL TURBO® DNase (2 U/µL, Life Technologies-Ambion, Burlington, ON) at 37 °C for 30 min. The transcribed RNA was purified using the RNeasy mini kit "RNA clean-up" protocol (Qiagen, Toronto, ON) and run as per manufacturer's specifications. Complete DNA removal was confirmed by PCR amplification of the digested material using a RT-PCR reaction with and without the RT component. RNA quantification was done using the Qubit® 2.0 Fluorometer system and RNA broad range kit (Life Technologies-Invetrogen, Burlington, ON). Copy number analysis was done using the following equation:

$$\frac{concentration(ng)\ x\ 6.022x10^{23}/mole}{base\ pair(bp)x\ 1x10^{9}\,{}^{ng}/_{g}\ x\ Molecular\ Weight\ bp\,{}^{g}/_{mole}}$$

In vitro transcribed FMDV RNA was done using PCR generated amplicons, rather than linearized plasmid. Template PCR amplicons for *in vitro* transcription were amplified in a 50 µL PCR reaction with the following reagents: 1.5 µL of M13 (-20) forward primer (5`-GTAAAACGACGGCCAG-3`, 50 µM) and M13 reverse primer (5`-CAGGAAACAGCTATGAC-3`, 50 µM), 10µL of 5x Phusion HF buffer, 1µL of dNTPs (10mM), 0.5 µL of polymerase (5 U/µL) and up to 50 µL of ultra-pure water and 1 µL of template RNA. The PCR reactions were ran on the Veriti thermocycler at the following conditions: 94 °C for 2 min, followed by 35 cycles of: 94 °C for 30 s, 50 °C for 1 min, 68 °C for 1 min and followed by a final extension of 68 °C for 5 min.

2.2.2. Primer and Probe Design

Literature searches were conducted initially to determine published genomic regions, primers and probes that have been used for detection of the target viruses. Genomic regions listed in Table 2.2 were selected because of a high degree of sequence conservation between known target sequences. Sequence databases were created from publically available sources such as the National Center for Biotechnology information (NCBI). Sequences were analyzed using an established bioinformatics pipeline routinely used by the lab. The pipeline includes sequence alignments with ClustalW (Larkin et al. 2007) and management of sequences (such as trimming and deletion of sequences) using Bioedit (Hall, 1999) and Excel. Sequence databases for FMDV, CSFV, ASFV, PCV2 and PRRSV were generated to produce primers and probes as well as accurately screen existing primers and probes against known sequences. Briefly, databases of approximately 2880 FMDV sequences, 800 CSFV sequences, 171 ASFV sequences, 538

PCV2 sequences and 658 PRRSV full and partial sequences were generated. Sequences were then analyzed, to generate other possible primers and probes under *in situ* conditions using a primer/probe design software such as AlleleID® v. 7.7 (Premier BioSoft Internation, Palo Alto, CA). Resulting oligomers were analyzed by "Basic local alignment search tool (BLAST)" to identify candidate oligonucleotide primers and probes. Candidate primers and probes were selected based on how well the sequences aligned to its specific target and whether it matches any non-specific sequences. Primers and probes for SVDV and VESV were selected from the literature based on previous work (Lung et al. 2011). A non-specific binding probe (NSBP), used as a negative control and to establish baselines for non-specific reactivity on the microarray, was also obtained from the literature (Hindson et al. 2008). All oligonucleotides were ordered from IDT at 25 nM scale for testing and resuspended in 1 x TE. Working Stocks of 50 µM and 10 µM were created for all primers and probes respectively, and stored at -20 °C until use.

2.2.3.0. RT-PCR Optimization

A panel of 16 primers was assembled from the literature search or designed after bioinformatics analysis. A seven-plex multiplex with an internal control was developed using the SuperScript® III OneStep RT-PCR with Platinum Taq kit. Using manufacturers suggestions; a 50 µL PCR reaction was made using the following reagents: 1 µL of the 16 specific primers (10 µM) for each individual target, 25 µL of 2x RT buffer mix, 2 µL of SSIII enzyme mix, 20-21 µL of ultra-pure water and 1 µL of target sample. Initial RT-

PCR conditions, run on the Veriti thermocycler, included: one cycle of 50 °C reverse transcription for 30 min and 94°C for 2 min followed by 40 cycles of 1 min of 94 °C, 1 min of 50 °C and 1.5 min of 68 °C, followed by one cycle of a final extension of 68°C for 5 min. Agarose gels made to 1% using UltraPure™ Agarose in UltraPure™ 10x TAE buffer (Thermo Scientific-Fermentas, Ottawa, ON) and mixed with 0.5ng/µL ethidium bromide (Sigma-Aldrich, St. Louis, MO), were used for visualization on the Gel Doc™ system (Bio-Rad, Mississauga, ON).

Primer concentrations were altered one at a time from a final concentration of 0.2 to 1 µM. Favorable concentrations of each primer, that best amplified of the target with the least negative effect on other target amplifications, were selected for further optimizations. After primer screening, RT-PCR running conditions were altered and compared. PCR running conditions tested include: RT temperature (50, 55 & 60 °C), RT time (15 & 30 min), denaturation time (2, 5, 7 & 10 min), annealing temperature (40, 45, 48 & 50 °C), annealing time (30 & 60 s) and cycling number (35 & 40).

To reduce non-specific amplification events, modified primers, dNTPs and master mix from TriLink Biotechnologies Inc. (San Diego, CA) were selected for testing. CleanAmp™ Precision primers and dNTPs are modified using a phosphotriester (PTE) group which work to hinder amplification until a high temperature is reached (Lebedev et al. 2008; Hidalgo Ashrafi, Yee, and Paul 2009). A 3`PTE group is added to each forward primer of the seven targets and then amplified in single-plex and in multiplex at standard concentrations. Amplicons were run on 1% agarose gels. The CleanAmp™ dNTPs were tested at standard concentration (0.2 mM final) and run using a 10x PCR buffer, supplied by TriLink, and the SSIII polymerase. TriLink supplied reagents to test their

CleanAmp™ One-Step RT-PCR 2x Master mix. The CleanAmp™ master mix was tested along side the standard assay using the same running conditions, with the exception of a 72 °C extension temperature.

2.2.3.1. Microarray Optimization

The microarray assay optimization was split into two components: Pre-treatment and Post-treatment. Pre-treatment conditions included addition of 1% sodium hydroxide (NaOH) and Lambda exonuclease (Thermo Scientific-Fermentas, Ottawa, ON) to keep the DNA in single strand form longer and to digest the anti-sense strand respectively. Post-treatment conditions included; reporter choice, reverse biasing and addressing time and amperage. All conditions were tested with and without the post treatment and run as per standard settings.

To optimize pre-treatment conditions, a solution of 1% NaOH was added to the PCR amplicon before addition to the plate. Samples were then heated at 95 °C for 5 min and then snap cooled on ice before addition to the plate. Testing of Lambda exonuclease required the PCR amplicons to be generated with 5`phosphorylated forward primers. A 25 µL reaction mixture of: 2.5 µL 10x Reaction buffer, 1 µL Lambda exonuclease and 21.5 µL of amplicon DNA, was set to incubate at 37 °C for 30 min and then heat inactivated at 75 °C for 15 min.

Optimization of the post-treatment conditions included the following; two reporter probes were tested for the detection of foot-and-mouth disease virus (FMDV), a Red Universal reporter (RUR) and a 5` Red Specific reporter (5RS). The 5RS is a

reporter that is specific to a region in the FMDV amplicon and will only react to FMDV, while the RUR is sequence specific to the tag sequence that is added to all of the reverse primers when amplifying the target pathogens. Each reporter probe was added separately, together, or one after the other to the plate before the run. Addressing times were altered from 120, 60 and 30 s, and addressing temperatures were also modified from 995, 800, 600 and 400 nA. Each time change was tested using each temperature setting until an optimal positive signal to negative background was reached. Reverse biasing (RB) is the use of a reverse current that will theoretically drive away any unbound targe DNA or reporter. The RB was tested using time intervals; 30, 10, 5, 2, 1 second and amperages from -995, -350, -150, -50 nA, at the end of the reporting stage of the microarray.

2.2.3.2. Seven-plex Multiplex RT-PCR

A multiplex RT-PCR was developed to amplify seven viruses that affect swine. The seven-plex RT-PCR with 22 primers (Table 2.3) was developed using SSIII OneStep RT-PCR with Platinum Taq kit. Each 50µL RT-PCR reaction consisted of the following reagents: 1 µL each of 1µM FMDV, CSFV and PCV2 primers (n=6) and 0.5µL each of 0.5 µM SVDV, VESV, ASFV and PRRSV primers (n=8), 25 µL of 2x RT buffer mix, 2 µL of SSIII enzyme mix, 15 µL of ultra-pure water and 1 µL of template. The RT-PCR reactions were performed with the Veriti thermal cycler using the following conditions: 55 °C RT for 15 min, then 94 °C for 2 min for initial denaturation, followed by 35 cycles of 94 °C for 30 min, 50 °C for 1 min and 68 °C for 1 min, followed by a 5 min final extension step at 68 °C.

Post-amplification reactions were visualized using one of two methods: The QIAxcel system was used for most samples, except for FMDV samples and clinical samples that were done on standard gel electrophoresis with SYBR® Safe (Life Technologies-Molecular Probes, Burlington, ON) in Winnipeg. The QIAxcel is an automated gel electrophoresis system that utilizes a multicapillary cartridge with agarose gel and small amounts of Ethidium Bromide (EtBr) to visualize the DNA. Briefly, post-PCR samples are loaded into a 12 slot tray, after which the machine is run using the AM320 settings. The cartridge draws up <0.1µL of sample and runs it through the gel contained within the cartridge, where a detector measures the excitation of the EtBr bound to the DNA by a LED light. Then a photomultiplier collects the data and the BioCalculator software calculates and creates the gel image. Mixtures of two size markers (15 and 3000 base pairs) are used in every run as a size reference. For conventional agarose gel electrophoresis, a 1% agarose gel is made with 1xTBE buffer, UltraPure™ Agarose and 2µL of SYBR® Safe. 5µL of amplicons are added to 1.5µL loading dye, loaded onto the gel and ran at 100 volts for 45 min and visualized using a Gel Dock system (Bio Rad, Mississauga, ON).

To determine the analytical sensitivity and to examine effects on amplification sensitivity, the RT-PCR assay was tested using three methods; single-plex with primers for single virus, seven-plex multiplex with all 22 primers, a five-plex with primers for the five exotic viruses (FMDV, SVDV, CSFV, ASFV, VESV), and a duplex assay for the two indigenous viruses (PCV2, PRRSV). All cycling conditions for each method are similar to seven-plex assay. PCR buffer compositions are also similar except for the

removal of appropriate primers (i.e. a single primer pair was used in the single-plex assay and five pairs of primers were used in the five-plex assay).

2.2.3.3. Electronic Microarray

The NanoChip 400 electronic microarray is an "Amplicon-to-Answer" system that automates and integrates all steps in microarray processing such as; probe printing, electrophoretically-driven hybridization, washing and reporting using one automated machine and disposable cartridges with 400 features. The NanoChip 400 platform consists of a small cartridge that contains an array of 400 test sites that are independently controlled by platinum electrodes covered in streptavidin containing polyacrylamide hydrogel (Papatheodorou et al. 2010). The protocol and methods for the NanoChip 400 assays were described previously (Lung et al. 2012; Papatheodorou et al. 2010). Briefly, a 96-well plate is set up to contain; 250 nM of each sequence specific biotin-labeled capture probes (Table 2.4) diluted in buffer mixture consisting of 50 mM Histidine (Sigma-Aldrich, St. Louis, MO) and 0.05% proclin® 300 (Sigma-Aldrich-Supelco, Bellefonte, PA), otherwise called "Hisproclin". A mixture of 8.75 µL post RT-PCR amplicons and 61.25 µL Cap-down A buffer (Nexogen, Inc., San Diego, CA) were added to the plate, as well as 2.8 µL of 1 µM fluorescent universal reporter probe in 67.2 µL High Salt Buffer (Nexogen, Inc., San Diego, CA). Samples were run in duplicate with a No Template Control (NTC) on each cartridge. A panel of virus-specific capture probes printed on the array includes at least two probes per target as well as the NSBP probe as a negative control to determine baseline non-specific reactivity.

At the start of the program, the NanoChip 400 instrument prints the capture probes to specific user-designated electrode-controlled test sites on the platinum array, using user defined parameters (350 nA of current for 30 s for this study). The array is then washed with a mixture of Hisproclin and 20% Triton X100 (Sigma-Aldrich, St.

Louis, MO) and then with milliQ water plus 0.05% proclin® solution five times. The target amplicons are then addressed to designated test sites for 60 s at 800 nA and then washed with Hisproclin/Triton mix and water. The universal reporter probe was added to the array at 60 °C, the temperature was lowered to 55°C for 60s and further reduced at 2 °C increments every 20 s until 25 °C. At this point High Salt Buffer (HSB) wash was applied and an image was taken at 24°C followed by another HSB wash and a final wash at 50°C. Three additional imaging steps were carried out at the end at different integration times; 1500, 1250 and 1000ms to allow selection of the image with optimal signal to noise ratio, if needed. Fluorescence data generated by the hybridization of the reporter probe to the bound target from the 1250ms images were used to calculate the Positive:Negative (PN) ratios. The PN ratios were calculated by dividing the specific target signal by the background signal generated by the NTC, which is run with each cartridge. An experimentally determined cut off of a PN ratio of two was used to determine whether a sample is positive or negative.

2.2.3.4. Initial Assay Validation

An initial panel of 58 laboratory amplified strains representing the diversity of the target viruses, including all known serotypes of FMDV (n=7) and genotypes of CSFV (n=3) were selected to validate the amplification and detection of the selected primers, probes and methods used in the developed assay. A collection of 11 oral and nasal clinical swab material from healthy hogs, as well as 11 non-target virus and bacteria that affect swine, were also tested to evaluate assay specificity, for a total of 22 negative

samples (Table 5). Overall, a total panel of 80 samples were used in the validation of the seven-plex assay.

The RT-PCR followed the above stated multiplex protocol (2.3.0.) and all samples were amplified within a 24 hour period to reduce variability from RNA degradation. The 22 negative samples were run twice; with and without a 1/50 dilution of PRRSV YNL RNA spiked in as a control to rule out the presence of inhibitors. All samples were run on QIAxcel for visualization. The standard NanoChip 400 microarray protocol was used for detection, followed by analysis.

2.2.4.0. Clinical Samples

2.2.4.1. Evaluation of Clinical Samples

From a list of over 1000 clinical samples, collected from the experimentally inoculated pigs, a panel of 114 samples were chosen for use in this study. The clinical samples for FMDV and SVDV were extracted using QIAamp viral RNA kit (Qiagen, Toronto, ON) and RNeasy mini kit (Qiagen, Toronto, ON) for CSFV and ASFV, as per manufacturer's specifications. The samples were evaluated first by using the animal care reports supplied by the animal care veterinarians to see when first signs of infection appeared, secondly by the seven-plex RT-PCR and thirdly by real time PCR for quantification. Amplicons generated were visualized using agarose gel and SYBR safe. NanoChip 400 conditions used for testing clinical samples are described below.

2.2.4.2. Experimental Inoculation (Spiking) into Clinical Material

Clinical samples containing VESV, PCV2 and PRRSV were not available for this study, thus lab amplified viruses were spiked into biological material from healthy animal to simulate clinical samples. Biological material used for spiking included oral and nasal material (Prairie Swine Centre, Saskatoon, SK). For PCV2 and PRRSV, 14 µL of laboratory-propagated virus ($1.4x10^{10}$ and $5.2x10^{9}$ $TCID_{50}$/mL, respectively) was spiked into 126 µL of biological material and was extracted with the RNEasy mini kit (Qiagen, Toronto, ON). For VESV, 20 µL of virus culture was spiked into 120 µL of clinical material and extracted with the QIAamp viral RNA kit. All extracted spiked samples were tested for the presence of target viruses with the seven-plex assay.

All post-PCR amplicons for inoculum and spiking were run on the NanoChip 400 using the optimized protocol. A total of six samples and a NTC (N=7) were run on each cartridge, in duplicate, against the full panel of specific and negative capture probes (n=28). Repeats were determined by selecting samples with unexpected or discordant results. For repeats, the amount of starting template material used was increased from 1 µL to 3 µL for samples that amplified, failed to amplify, and samples with low microarray signal were repeated by increasing the amount of amplicon added to the array from 8.75 µL to 16 µL.

2.2.5. Limit of Detection: Analytical Sensitivity

In vitro transcribed RNA was used to determine the analytical sensitivity of the assay for each target. After quantification, *in vitro* transcribed RNA was diluted serially from neat stock to 10^{-12} in dH2O. The serial dilution mixture consisted of 18 µL dH2O and 2µL neat RNA sample (FMDV=$1.9x10^{12}$, SVDV=$1.2x10^{12}$, CSFV=$3.5x10^{11}$, VESV=$6.4x10^{11}$, ASFV=$1.4x10^{10}$, PCV 2=$3.9x10^{10}$ and PRRSV=$1.1x10^{12}$ copy number/µL) for a 10-fold dilution and the process was repeated 11 more times to create the full dilution series. The dilutions were all made on the same day they were used. To amplify, 1 µL of the dilution series was amplified using the standard seven-plex multiplex, five-plex exotic, two-plex indigenous and single-plex RT-PCRs and imaged using the QIAxcel. RT-PCR end points were determined by the last reaction in the series to give a visually detectable band. To determine the last detectable band, an arbitrary threshold cut-off set by the QIAxcel program on the electropherograph was used; presence of a band of the expected size with signal intensity above the threshold were categorized as positive, while an intensity below the threshold was considered negative.

A panel of six samples from each dilution series including; two samples above the RT-PCR endpoint and three samples past the endpoint, were chosen to run on the NanoChip 400 assay with a positive control and NTC (n=8) in duplicate on each cartridge. The eight samples were run against a reduced panel of detection probes that included only the specific target probes (ie. FMDV samples tested against the five FMDV probes) as well as the NSBP as a negative control probe. The optimized NanoChip 400 assay was used throughout the experiment. The PN ratio cutoff for positivity of 2 was used to determine the analytical sensitivity of the microarray assay. PN ratios ≥ 1.7 and ≤ 2 were designated as suspect.

2.3.0. Results

2.3.1.1. Multiplex RT-PCR development

A multiplex RT-PCR assay was developed to target seven high consequence viruses that affect swine. A final set of 22 primers was selected to amplify the seven viruses (Table 2.3). Primers were chosen because of their selective reactivity to the target virus as seen in Figure 2.1. The seven-plex RT-PCR amplified the target viruses, including all 7 serotypes of FMDV, 3 genotypes of CSFV, both genotypes 1 (EU) and 2 (NA) of PRRSV. All targets generated amplicons of the expected size. Oral material from healthy animals and No-Template Control (NTC) did not produce any amplification product (Figure 2.1).

2.3.1.1.1. Seven-plex RT-PCR Multiplex Optimization

Optimization of the primer concentrations showed that the FMDV and CSFV amplified its targets best at 1 µM primer concentration while the other primers amplified most specifically at 0.5 µM. After determination of primer concentrations, cycling conditions such as; RT time and temperature, initial denaturation time, annealing time and temperature, and cycling number were tested. Results showed that increasing the denaturation and RT time had little effect on amplification while increasing the RT temperature to 55 °C did increase the specificity of amplification for FMDV (data not shown).

Modified primers from Trilink were evaluated to determine whether it could increase the specificity of the seven-plex RT-PCR. In single-plex, the modified primers showed lower band intensities than the non-modified primers for FMDV, CSFV, VESV and PRRSV. No amplification was seen for ASFV and PCV 2 with the CleanAmp primers, while SVDV was the only sample to show higher intensities than control unmodified primers (Figure 2.2). No bands were detected when the full set of CleanAmp™ primers were used for the seven-plex RT-PCR with or without using the CleanAmp™ dNTPs (data not shown).

2.3.1.1.2. Seven-plex RT-PCR Validation

A panel of 58 viral samples of the seven target viruses (Table 2.5) were amplified with the optimized seven-plex RT-PCR. Panels of the target viruses were selected from available laboratory strains that represent the diversity of strains and included all seven FMDV serotypes and 3 genotypes of CSFV. A full FMDV panel of 23 strains were amplified successfully along with 12 SVDV, 10 CSFV, one VESV, three ASFV, one PCV 2 and eight PRRSV strains.

The FMDV samples showed non-specific amplification that was weaker than the specific FMDV amplicons (Figure 2.3A). All 35 non-FMDV isolates of target viruses amplified well, with minimal non-specific bands (Figure 2.3B).

A total of 11 oral and nasal swabs from healthy pigs as well as 11 non-target swine bacteria and viruses were used to assess the specificity of the amplification.

Twenty one of the 22 samples used to test specificity did not result in detection of amplified products unless the sample was spiked with 1 µL of 1/50 dilution of PRRSV YNL RNA as an exogenous control. With the *Streptococcus suis* sample, some non-specific banding was observed possibly due to non-specific primer binding. The exogenous control, spiked PRRSV, was detected, indicating there is no detectable level of PCR inhibition (Figure 2.3C & 2.3D).

2.3.1.2. NanoChip 400 Electronic Microarray

2.3.1.2.1. NanoChip 400 Optimization

RT-PCR amplicons were transferred to the NanoChip 400 electronic microarray for screening of the 46 capture probes designed for detection of the target viruses. The NanoChip 400 was optimized with the following conditions separately; addressing conditions, wash buffer, reporter probes and pre/post-treatment of amplicons. Amplicons were treated with either; 1% NaOH, heat denaturing, or lambda exonuclease prior to addition to NanoChip 400 array. NaOH and lambda exonuclease showed similar or lower fluorescence values than the non-treated controls (data not shown). Results for the addressing conditions showed that 800nA for 60 s was optimal and produced the highest fluorescent signals to background (data not shown). Testing of two different reporters probes showed that the 5RS reporter had higher fluorescent signals and lower backgrounds than the RUR reporter. RUR reporter generated PN ratios for positive signals about 2 PN levels lower than the 5RS reporter as well as showed higher background. All fluorescent signals were lost after all RB treatments, where the

electrodes were given a negative charge as compared to the regular positive charge, such as -995 nA for 30 s. The only exception was -50 nA for 1 second, which generated weak results compared to the non-RB data (data not shown).

Following optimization, 27 capture probes, at least two probes per target, were selected along with a negative control non-specific binding probe (Table 2.4). The panel of 28 probes, the seven virus amplicons and a NTC sample were addressed to the microarray using the optimized protocols. All targets reacted as expected with their respective capture probes whereas the probes for PRRSV EU and NSBP remain unreactive due to lack of specific targets to bind to (Figure 2.4).

2.3.1.2.2. NanoChip 400 Electronic Microarray Validation

The panel of 58 validation isolates and 22 negative control samples were tested in duplicate against the select panel of 27 capture probes and one negative binding probe using the optimized protocol. All probes gave good fluorescent sensitivity to their target virus without any cross reactivity to non-target samples. All negative control samples showed no cross reactivity (Figure 2.5).

2.3.2. Testing with Clinical Samples

A total of 126 clinical samples, collected from experimentally infected pigs, were chosen for extraction and evaluation using the seven-plex multiplex assay. Most of the samples tested all detected to comparable days post-infection (dpi) levels with both the

real time and seven-plex RT-PCR. However, FMDV RNA in serum samples were detected as early as dpi 1 whereas a real time RT-PCR used by the reference laboratory in Winnipeg first detected the virus at dpi 2. Targets were detected in a variety of sample types including; nasal, oral, serum and whole blood. First detection ranging from 1 dpi for SVDV, 4 dpi for ASFV and 5 dpi for CSFV (Table 2.6). For VESV, PCV 2 and PRRSV, virus culture was experimentally inoculated into oral and nasal samples were successfully detected (Table 2.6).

2.3.3. Limit of Detection: Analytical Sensitivity

Figure 2.6 depicts the amplification of SVDV ITL 19/99 transcribed RNA using the three different RT-PCR tests. The target was amplified out to the $1x10^{-8}$ dilution for single-plex and five-plex multiplex and $1x10^{-7}$ for the seven-plex RT-PCR. All other virus dilution series amplified well with the three RT-PCRs and detected using the NanoChip 400 (Table 2.7). The sensitivity of the assay was best for ASFV, PCV 2 and PRRSV at approximately 1 copy by PCR and 10 copies by microarray, followed by SVDV, CSFV and VESV at around 10 and 100 copies by PCR and microarray respectively. The lowest detection was observed for FMDV at around 100 copies by PCR and 1000 copies by microarray. In general, the single-plex and five-plex RT-PCRs, had a sensitivity that was 10 fold better than the seven-plex RT-PCR assay (Table 2.7).

2.4.0. Discussion

The seven viruses chosen for this project represent a threat to the Canadian livestock industry, economy and ecosystem. With this in mind, the government of Canada has implemented procedures and validated tests to detect and protect against these pathogens as prescribed by the OIE 'Manual of Diagnostic Tests and Vaccines for Terrestrial Animals'. The main problem facing the diagnosis of these different viruses are shared clinical signs such as; the vesicular lesions caused by SVDV and VESV being clinically indistinguishable from FMDV (Banér et al. 2007). The purpose of this project was to develop a user-friendly diagnostic assay with the ability to detect and differentiate between seven high consequence swine viruses in a single reaction using a multiplex RT-PCR paired with an automated NanoChip 400 electronic microarray.

Optimization of the assay stemmed from the complex interactions between the primers, amplicons and the probes. A Dengue virus internal control was removed from the assay due to its strong heterologous reaction with FMDV and PRRSV primers and once removed; stronger amplification of FMDV and PRRSV was observed (data not shown). Homologous primer interactions also pose a challenge to assay sensitivity by reducing the amount of available primers in the reaction (Yuryev 2007). Though the PCR and microarray assays were optimized for this project, further optimizations may improve the amplification, sensitivity and turn around time. Optimizations including more efficient enzymes, reaction buffers or chemical modifications have the potential to increase sensitivity of the assay. One optimization that was evaluated was the TriLink Biotechnologies' CleanAmp™ primers and dNTPs. The CleanAmp™ system uses a chemical modification on the forward primer to block the DNA polymerase during the initial reverse transcription step to reduce possible mispriming (Shum and Paul 2009;

Hidalgo Ashrafi, Yee, and Paul 2009). Kits and modifications such as these have the potential to enhance amplification of single or multiple targets. The CleanAmp™ primers and dNTPs tested in this project only produced amplicons in single-plex reactions, but not in multiplex reactions (Fig 2.2). One explanation for why the multiplex PCR failed may be due to high DMSO in the buffer used to suspend the primers. DMSO is used to promote DNA separation in regions of high GC content (Jensen, Fukushima, and Davis 2010). When the primers are diluted to at least 0.5 µM in H_2O, the DMSO concentration is diluted 400x. This allowed the single-plex reaction to work, but in a multiplex reaction with seven modified primers, the concentration of DMSO is 36x the original concentration. Though the CleanAmp™ system didn't work in a multiplex system, the primers may be useful by introducing the modified primers for problem samples that are highly reactive to non-specific oligonucleotide sequences.

The developed RT-PCR amplified a panel of 58 viral strains, representing all seven high consequence viruses, with serotype and genotype representations for viruses such as FMDV (S. M. Reid et al. 2002) and CSFV (Dirk Deregt et al. 2006). The RT-PCR did not show significant non-specific amplification of 11 clinical materials from healthy pigs and 11 non-target viruses and bacteria associated with livestock. Some weak non-specific amplifications representing mispriming of the 22 primers with the sample, are non-reactive against the capture probes in the microarray. Amplicon mispriming could potentially lower assay sensitivity by reducing amplification of specific targets, creating a competitive PCR (Peyrefitte et al. 2003). Mispriming can be reduced by incorporating *in silico* analysis and avoiding primers that bind to host or relevant non-target sequences.

For optimizing the microarray assay, a 1% NaOH amplicon denaturing step was added prior to addition to the microarray. Since the microarray only requires the anti-sense strand of the amplicon containing the reporter tag sequence to hybridize to the capture probe, it was hypothesized that this treatment would increase sensitivity, by increasing specific annealling of the target DNA and capture probes. The lack of affect maybe due to the low NaOH concentration. One explaination could be that the change in NaOH changed the electro competence of the solution, or the electrical currents potential to travel through a liquid medium. The Cap down A buffer (CdA) that is used to suspend the amplicons needs to be between 0 and 100 µS/cm to allow for correct current travel, and the addition of the NaOH changes the competence of the buffer (data not shown). A solution containing 1% NaOH was the maximum percentage that would result in a buffer with the required 0-100 µS/cm range. A lambda exonuclease step was evaluated to digest any DNA strand that has a 5` phosphate, leaving the tagged antisense strand for hybridization (Lung et al. 2015). Though a single stranded product was being produced, it is possible that it wasn't enough to increase the efficiency of hybridization as compared to the no-treatment control. Another possibility is that the addition of the exonuclease reaction, buffer and exonuclease, was reducing hybridization efficiency.

Reverse bias in theory can aid washing by electrophoretically driving any unbound amplicons away from the array surface and the probes by giving it a negative charge. Experimentally, it was observed that at most of the conditions tested, most or all signal is lost suggesting that the reverse current is too strong and prevented hybridization of the target amplicon with the capture probes or annealing of the fluorescent reporter with the tag sequence on the amplicon. At low amperages and times, a significant amount

of signal remained after RB, though it was not as high as the no-RB control (data not shown).

Using clinical material, the RT-PCR and microarray assays detected viral nucleic acid as early as 1 dpi in serum from animals experimentally inoculated with FMDV, as well as in other sample types (ie, oral and nasal swabs, and whole blood). Detection from the samples varied widely depending on the status of the infection. One experimental infection of ASFV failed to show clinical signs until day 13 due to a late start to the infection seen when the animal failed to show symptoms until days after inoculation (animal care notes, data not shown). Due to the lack of clinical samples for VESV, PCV 2 and PRRSV, these viruses were spiked into biological material collected from healthy ≥2 day old hogs. Detection of viral nucleic acid in spiked samples showed that the assay is able to detect these viruses in biological material.

The analytical sensitivity of the seven-plex, five-plex exotic and 2-plex indigenous RT-PCRs were determined using quantified *in vitro* transcribed RNA. The lowest limits of detection observed for the seven-plex RT-PCR were for PRRSV, PCV 2 and ASFV. These three viruses have the smallest amplicons (379-537bp). Two of the viruses with small amplicon size are DNA viruses, which make the RT step during PCR irrelevant. The use of the RT step with the DNA viruses may make the amplification more efficient in comparison to the larger amplicons from the RNA viruses. This could be due to the fact that present in clinical samples, the DNA virus may have both DNA and messenger RNA strands present for amplification, while the RNA viruses will have just the messenger RNA and genomic RNA, but no initial DNA present, giving a possible detection advantage to the DNA viruses.

Due to the unavailability of virus stocks in Lethbridge, the *in vitro* transcribed RNA was used as a facsimile for the five RNA viruses present in the multiplex. Though the use of real virus stocks to determine the analytical sensitivity would have been preferable to only a small region of the whole virus genome, it is difficult to accurately quantify the virus samples propagated *in vitro* due to high levels of secondary structure (Plaskon, Adelman, and Myles 2009) or by random-priming of cellular or exogenous small nucleic acids (Timofeeva and Skrypina 2001) that are released during extraction. Using *In vitro* transcribed RNA is favourable also because it has a higher degree of reproducibility during quantification and testing (Schibler et al. 2012).

The limit of detection (LOD) for FMDV was lower than the other targets. The most likely reason for this lower sensitivity is due to the size of the amplicon. The selected FMDV amplicon was 981bp long to allow simultaneous serotyping. The VP1 capsid protein-coding region is a highly variable region making it ideal for subtyping FMDV (Carrillo et al. 2007). It has been documented that larger amplicons generated by PCR produce lower yields than shorter PCR products due to a few possible reasons. One reason for the lower yield could be 3`-terminal base mismatching in primer binding, causing early termination or hindering strand synthesis (Cheng et al. 1994). The location of the mismatch is important if the mismatched nucleotide is located within the last four nucleotides from the 3`-terminus, the reaction yield is either greatly reduced or fails to amplify. Similar reactions were seen by other researchers that saw reduced amplifications depending on what kind of mismatch was present (Huang, Arnheim, and Goodman 1992). Another issue that may be affecting the LOD can be the competition between the FMDV and other primers in the assay. It was seen earlier in the project that the Dengue

virus internal control sequence, was forming primer dimers on multiple primers in the assay, FMDV and PRRSV being one (data not shown). After removing the Dengue primers from the assay, amplification for the affected targets increased. There may be a similar effect happening between the FMDV primers and another set of the primers in the assay. All work with the FMDV samples was done quickly and completed, from serial dilution and quantification to microarray within a two-day span. This helps reduce the variation that may occur due to freeze-thaw cycles, causing degradation of the RNA that would decrease amplification efficiency. Another possible cause could be the presence of PCR inhibitors that affect amplification of larger targets more than small ones. With the likely culprit of the low FMDV sensitivity being its amplicon size, future work on reducing the amplicon size by either reducing within the current genetic region finding another conserved region may facilitate a more efficient reaction.

Electronic microarrays are a powerful tool for detection and typing of pathogens. The process of using an electrical current greatly increases the level of detection and the speed of hybridization by driving the negatively charged nucleic acid amplicons towards the probe bound test site in milliseconds compared to normal passive hybridization (Miller and Tang 2009) which relies on diffusion and can take hours to hybridize efficiently. Microarrays have been integrated into both human and veterinary medicine in the attempt to develop rapid and accurate point of care (POC) diagnostic tests. These POC tests are described as a near-patient test that is utilized in either a hospital setting for testing by medical personnel (S. Park et al. 2011; Niemz, Ferguson, and Boyle 2011) or pen side by a veterinarian (Bollo 2007). This gives the professional the greatest ability to rapidly diagnose diseases. Though a large benchtop system such as the NanoChip 400 is

less suitable for use as a POC machine, the technology used in the systems can be used in more mobile and cost effective instrumentation that can reduce both time and costs in POC lab-on-chip instruments (Tan et al. 2014; van Reenen et al. 2014) and Rheonix EncompassMDx workstation and disposable CARD® cartridges (Spizz et al. 2012).

Diagnostic systems such as the one described here has potential to complement existing tests and contribute to the economic stability of Canada. Gold standard tests have all been used in the detection and diagnosis of FAD outbreaks from VI for FMDV, ELISA assays for CSFV, virus neutralization for SVDV, indirect fluorescent antibody test for ASFV or Real-time PCR for VESV, PCV 2 and PRRSV (Table 1). These tests are described on the OIE website: http://www.oie.int/. These methods have been selected due to their sensitivity of detection by having distinct tests and antibodies for the different serotypes (OIE - World Organisation for Animal Health 2012, 2:149). Examples include the seven serotypes of FMDV and certain ELISA tests, such as the 3AB1 non-structural protein ELISA, have been shown to differentiate between infected samples and samples from vaccinated animals (Jaworski et al. 2011).

However, the problem with an ELISA test is that it requires high viral loads and specific sample types to accurately identify a true positive sample. If any sample is suspected of having too low of titres or the sample is an unusable type, such as milk or clotted blood, the sample must be sent for cell culturing which may take up to four days (S. Reid et al. 2003). Likewise other gold standard tests, such as VI and complement fixation, can hinder rapid diagnosis based on their nature. Virus isolation requires long incubation times of up to typically 4-6 days and complement fixation is complex and can introduce false biases into the results due to anti-complex formations (Ferris and Dawson

1988). Tests such as these require highly trained technicians and specialized equipment, leading to increased cost. These tests are also hindered by their low throughput, which in an outbreak situation where hundreds of samples need to be processed quickly, valuable time is spent on laboratory confirmation using single target tests. Laboratory confirmation of samples is important to confirm that the target pathogen is present or if there is any chance of another virus as the causative agent. An example of this can be from a possible FMDV outbreak where laboratory test must not only confirm the presence of FMDV but also rule out other viruses such as SVDV and VSV that cause similar clinical signs (Núñez et al. 1998; Lung et al. 2011; Fernández et al. 2008; Banér et al. 2007). The elapsed time in laboratory confirmation puts the surrounding areas and affected region in jeopardy of the spread of infection before any level of quarantine can be implemented.

The NanoChip 400 takes many of these issues into account, from the sensitivity to the throughput. The use of the electronic currents both increases sensitivity of low template samples as well as reduces wait times. With the use of the 400 individually controlled test sites; as many as 400 samples can be tested against one probe on a single cartridge. With the inclusion of multiplex RT-PCR technology, the NanoChip 400 is able to accurately detect multiple targets from a single amplified sample. Though the system can aid in diagnostic testing, one drawback is the reliance on separate DNA/RNA extractions and RT-PCR amplification to generate the appropriate template to run in the instrument. The separate extractions and RT-PCR require trained technicians to complete as well as add processing time to the test; thereby, increasing the chance of contamination with any handling steps. With systems that offer a rapid diagnosis and high throughput,

quarantine of affected farms and geographical areas can be put in place faster, halting the possible spread of infection. The combination of multiplex RT-PCR assays and electronic microarray technology offers this rapid and sensitive diagnostic capability by reducing the number of different tests that need to be run and decreasing the time between sample submission and test results. Savyon Diagnostics (Ashdod, Israel) has developed a new NanoChip 400 system call the NanoChip 400 XL which incorporates a PCR component into the workflow of the machine, reducing the manual handling steps required (https://www.savyondiagnostics.com/).

In conclusion, the “amplicon-to-answer” seven-plex RT-PCR and associated microarray assay was able to specifically amplify, detect and differentiate between seven high consequence swine viruses. Further testing is desirable to further optimize the RT-PCR. This project will be used as a starting point for integration onto advanced “sample-to-answer” systems to further increase the speed and automation to rapidly differentiate viruses.

2.5.0. Table Legend:

Table 2.1. List of gold standard tests prescribed by OIE for identification of selected viral pathogens

Virus	Gold Standard Tests				
	Virus Isolation	PCR/ TaqMan®	Virus Neutralization	ELISA	Other
foot-and-mouth disease virus [a]	+	+	✓	✓	Complement Fixation
swine vesicular disease virus [b]	+	+	✓	+	N/A
classical swine fever virus [c]	+	+	N/A	✓	Neutralizing peroxidase-linked assay (NPLA)
African swine fever virus [d]	+	+	N/A	✓	Indirect fluorescent antibody test (IFAT)
vesicular exanthema of swine virus *	+	+	N/A	+	Complement Fixation
porcine circovirus type II**	N/A	+	N/A	N/A	In situ Hybridization
porcine reproductive and respiratory syndrome virus [e]	+	+	+	+	Immunofluorescence assay

N/A = Not applicable
+ = Test available
✓ = Test prescribed by OIE

OIE – World Organization for Animal Health 2017: 2.1.8 [a], 2.8.8 [b], 2.8.3 [c], 2.8.1 [d], 2.8.6 [e]
OIE – World Organization for Animal Health 2017: 2.8.8 [b], 2.8.3 [c], 2.8.1 [d], 2.8.6 [e]
Zimmerman, et al. 2012: 493*, 412-413**

Table 2.2. List of five foreign and two indigenous viruses that affect swine targeted in Erickson et al. 2017

Virus	Abbreviation	Family	Genome Type	Gene Target	Clinical Signs
foot-and-mouth disease virus	FMDV	*Picornaviridae*	+ssRNA	VP3/VP1/2A/2B	vesicular lesions [a]
swine vesicular disease virus	SVDV	*Picornaviridae*	+ssRNA	3C/3D	vesicular lesions [b]
classical swine fever virus	CSFV	*Flaviviridae*	+ssRNA	E1/E2	hemorrhages, immunosuppression, lesions [c]
African swine fever virus	ASFV	*Asfarviridae*	dsDNA	VP72	hemorrhages, lesions [d]
vesicular exanthema of swine virus	VESV	*Caliciviridae*	+ssRNA	Polymerase	vesicular lesions [e]
porcine circovirus type II	PCV2	*Circoviridae*	ssDNA	Capsid	immunosuppression, lesions [f]
porcine reproductive and respiratory syndrome virus	PRRSV	*Arteriviridae*	+ssRNA	Matrix	reproductive failure, respiratory dysfunction, lesions [g]

Zimmerman et al. 2012 pgs 597[a], 604[b], 541[c], 399[d], 494[e], 410[f], 468[g]

Table 2.3. PCR primers used in Erickson et al. 2017 for the amplification of seven high consequence viruses

Virus	Genomic Region	Primer	Sequence (5`-3`)	Amplicon size (bp)	Reference
FMDV	VP3/VP1/2A/2B	VP3com980(1)	GCT GAT TAC GCG TAC AC	971	Erickson et al. 2017
		VP3com980(2)	GCT GAC TAC GCG TAC AC		Erickson et al. 2017
		VP3com980(3)	GCG GAT TAC GCC TAC AC		Erickson et al. 2017
		VP3com980(4)	GCG GAT TAC GCG TAC AC		Erickson et al. 2017
		VP3com980(5)	GCA GAT TAC GCG TAT AC		Erickson et al. 2017
		VP3com980(6)	GCA GAC TTT GCA TAC AC		Erickson et al. 2017
		VP3com980(7)	AGT GAC TTC TCC TAC AC		Erickson et al. 2017
		VP3com980(8)	GCT GAC TAT GCT TAC AC		Erickson et al. 2017
		VP3com980(9)	GCA GAC TTT GCC TAY AC		Erickson et al. 2017
		FMDV 2B Rev 4026-S-Deg2*	GCG GAC ACC ARC CGG TTR AAG TC		Erickson et al. 2017
SVDV	3C/3D	SVDVCV-3C-17a-F-(5875bp)	CAG CGG CAC TCC TCA GAC ACT AC	791	Lung et al. 2011
		SVDVCV-3D-3a-R-(6642bp)*	GAG TTT CAG GCA CGT AAA CCA CAC		Lung et al. 2011
CSFV	E1/E2	KBH12-5 E1 Ext FWD	AGR CCA GAC TGG TGG CCN TAY GA	671	Paton et al. 2000
		KBH12-6 E2 Ext REV*	TTY ACC ACT TCT GTT CTC A		Paton et al. 2000
ASFV	VP72	King Long – Fwd Primer	ATA GGA TTA AAA CCT ACC TGG AAC ATC TCC G	537	King et al. 2003
		King Long – Rev Primer*	GGT ACT GTA ACG CAG CAC AGC TGA ACC GTT CTG		King et al. 2003
VESV	Polymerase	VESVSM-2-F-(5101bp)	CGA CTC GAT GGA CCT GTT CAC ATA CG	649	Lung et al. 2011
		VESVSM-5-R-(5749bp)*	CGT AGA GGT CGG TTA GGT CCT TTC TG		Lung et al. 2011
PCV 2	Capsid	CircoV-1222F	GTA ATC AAT AGT GGA ATC TAG GAC	534	Lung et al. 2017
		CircoV -1760R*	TTC GTT TTC AGA TAT GAC GTA TC		Lung et al. 2017
PRRSV	Matrix	PRRS-Mtrx- F2	AAG GTA AGT CGC GGC CGA C	379	Lung et al. 2017
		PRRS-Mtrx -R2*	TGC CRC CCA ACA CGA GGC		Lung et al. 2017

* Reverse primers contain a complimentary tag sequence for Red Universal Reporter probe at the 5` end (Huang et al., 2009).

Table 2.4. List of capture probes used in Erickson et al. 2017 for printing on NanoChip 400 microarray

Virus	Genomic Region	Probe	Sequence (5`biotinylation* - 3`)	Reference
FMDV	VP3/VP1/2A/2B	FMD Common A	AAG TTG GCN GGA GAC GTB GAG TCC AAC CC	Erickson et al. 2017
		FMD Common B	AAC TTY GAC CTG TTA AAG TTG GCB GGA GAC GTT GAG TC	Erickson et al. 2017
		FMD Common C	AAC TTC GAC CTG TTA AAG TTG GCY GGA GAC GTT GAG TCC AAC CCT	Erickson et al. 2017
		FMD Common D	GAG TCC AAC CCT GGG CCY TTC TTC TTC	Erickson et al. 2017
		FMD Common E	GAG AYG TBG AGT CCA ACC CTG GGC CYT T	Erickson et al. 2017
SVDV	3C-3D	SVDV-020-deg	AAG AGA CAT YCT ATC CAA GAA GAC CAG AGA CCT TRC CA	Lung et al. 2011
		SVDV-014a	GGG TAG CGC CGT TGG GTG TGA CC	Lung et al. 2011
		SVDV-008-deg	GTG GCY YTG GGT ATC AAG AAA AGA GAC AT	Lung et al. 2011
		SVDV-013	GCA ATG AGG CAG ACA TTT GGA AAC CTA TA	Lung et al. 2011
		SVDV-016	AAA GAG ACA TCC TAT CCA AGA AGA CCA GAG ACC T	Lung et al. 2011
		SVDV-010-deg	TAT GGT CTA AAC YTR CCA ATG GTA ACC TA	Lung et al. 2011
		SVDV-019-deg	ACA ACT AGC CAC ACT RGA CAT YAG CAC KGA RC	Lung et al. 2011
CSFV	E1/E2	CSFV Common 1	CTT AAK GTG GTY AGT AGG AGG TAY	Erickson et al. 2017
		CSFV Common 4a	CTG RAY GAC GGR ACY GTY AR	Erickson et al. 2017
		bioKBH12-8 E2 Int FWD	TCR WCA ACC AAY GAG ATA GGG	Paton et al. 2000
		bioKBH12-9 E2 Int REV	GAT GAC TTY GGR TTY GGR CTG TG	Paton et al. 2000
ASFV	VP72	bioASFV-VP72-1668	CTG CTC ATG GTA TCA ATC TTA TCG A	King et al. 2003
		bioASFV-VP72-1898	ACG GCY GAT CTT GTG GTA TC	King et al. 2003
VESV	Polymerase	VESV-SM-010-deg	CCA CYA TGG CTA CTA CTC AYA CGC TTC TGT CGT TTG AC	Lung et al. 2011
		VESV-SM-003a-deg	CGG ATG CTG ARA TAA CGC CTA TCC C	Lung et al. 2011
PCV 2	Capsid	CircoV-1576	ATA TCC GAA GGT GCG GGA T	Lung et al. 2017
		CircoV-1657	GAC GAG CCA GGG GCG GCG GC	Lung et al. 2017
PRRSV	Matrix	PRRS 322-346 COM	TAC ATT CTG GCC CCT GCC CAT CAC G	Lung et al. 2017
		PRRSV-M-378	GGC AAA TGA TAA CCA CGC ATT TG	Lung et al. 2017
		PRRSV-M-361	GGC TTT CAT CCG ATT GCG GCA AAT G	Lung et al. 2017
		PRRS 233-252 EU	TTG TCA CCC TTC TGT GGG GC	Lung et al. 2017
		PRRS 380-402 EU	CGT CTG GTA ACC GAG CAT ACG CT	Lung et al. 2017
Non-Specific Binding		NSBP	CAA AGT GGG AGA CGT CGT TG	Hindson et al. 2008

* Probes modified with 5` biotinylation for binding to streptavidin pad on NanoChip 400 microarray (Takahashi, et al. 2008)

Table 2.5. Strains of the targeted swine viruses used in this Study

Virus	Genotype/ Serotype	Strain Name	Country of Origin
FMDV	A	Iran 1/96 (FMD 1)	Iran
		Arg 2/2001 (FMD 2)	Argentina
		Arg /87 (FMD 3)	Argentina
		COL /85 (FMD 4)	Columbia
		Iran/99 (FMD 5)	Iran
		Iraq 24/64 (FMD 6)	Iraq
		Cruzeiro/Bra/55 (FMD 7)	Brazil
	O	Manisa (FMD 8)	Turkey
		TAW 10/97 (FMD 9)	Taiwan
		UKG 11/2011 (FMD 10)	UK
		BFS /860 (FMD 11)	UK
	C	Noville (FMD 12)	Switzerland
		Resende (FMD 13)	Brazil
	Asia	PAK 1/54 (FMD 14)	Pakistan
		Shamir (FMD 15)	Israel
	SAT1	KEN 4/98 (FMD 16)	Kenya
		BOT 1/68 (FMD 17)	Botswana
	SAT2	ZIM 5/81 (FMD 18)	Zimbabwe
		SWA 1/69 (FMD 19)	Swaziland
		SAU 1/2000 (FMD 20)	Saudi Arabia
		ZIM 10/91 (FMD 21)	Zimbabwe
	SAT3	BEC 1/65 (FMD 22) a	Botswana
		ZIM 4/81 (FMD 23)	Zimbabwe
SVDV		GRE 1/79	Greece
		FRA 1/73	France
		HKN 1/80	Hong Kong
		HKN 3/89	Hong Kong
		ITL 1/66	Italy
		ITL 1/97	Italy
		JAP 1/74	Japan
		NET 3/92	Netherlands
		PORT 1/2003	Portugal
		SWI 1/74	Switzerland
		UK27/72	UK
		ITL 19/92 a	Italy

a - Stains used in the development
b - Synthetic DNA construct

Table 2.5. Continued

Virus	Genotype/ Serotype	Strain Name	Country of Origin
CSFV	1.1	Alfort/187 a	France
	1.2	Brescia	Italy
	1.3	VRI 4167	Malaysia
	2.1	NL B64	Spain
	2.2	Vi 3295/4/89	Germany
	2.3	Diepholz 1/Han94	Germany
	3.1	Congenital Tremor	UK
	3.2	3.2 b	USA
	3.3	3.3 b	USA
	3.4	Kanagawa (Tap 3)	Japan
ASFV	1	Lisbon-61 a	Portugal
	1	Lillie	France
	2	Georgia 2007	Georgia
VESV		VESV-CAL a	USA
PCV2	Type 2	PCV2 B a	Canada
PRRSV	2	PRRS 2.5	Canada
	2	PRRS Vaccine 2.5	Canada
	2	PRRS 1.4	Canada
	2	PRRS MLV June 2004	Canada
	2	PRRSV Vac 2,5	Canada
	2	PRRSV P-YNL a	Canada
	2	PRRSV 93 44927	Canada
	1	PRRSV LV	

a - Stains used in the development

b - Synthetic DNA construct

Table 2.6. Testing of assay with clinical and spiked biological samples

Virus	Serotype/Genotype	No. of Animals	No. of Samples	Sample Type	Electronic microarray	Real Time PCR
FMDV	A IRN 1/2009, O UKG 1/2011, SAT1 ZAM 9/2008		14	Serum	Earliest detection at dpi of 1	Earliest detection at dpi of 2
	O UKG 1/2011		6	Oral	Earliest detection at dpi of 2	Earliest detection at dpi of 1
	O UKG 1/2011, SAT1 ZAM 9/2008		10	Nasal	Earliest detection at dpi of 2	Earliest detection at dpi of 1
SVDV	PORT 1/2003, UK27/72	4	36	Nasal	Earliest detection at 1 dpi	Earliest detection at 1 dpi
CSFV	Diepholz, Honduras	4	36	Serum	Earliest detection at 5 dpi	Earliest detection at 5 dpi
ASFV	Malawi	4	24	Whole Blood	Earliest detection at 4 dpi	Earliest detection at 4 dpi
VESV*	Cal	-	1	Oral	Detected	N/A
		-	1	Nasal	Detected	N/A
PCV 2*	Weirrenga	-	1	Oral	Detected	N/A
		-	1	Nasal	Detected	N/A
PRRSV*	YNL (NA), LV (EU)	-	2	Oral	Detected	N/A
	YNL (NA), LV (EU)	-	2	Nasal	Detected	N/A

* Clinical samples spiked with live virus

Table 2.7. Analytical sensitivity of the RT-PCR and Electronic Microarray Assay

		Assay Detection							
		RT-PCR				Electronic Microarray			
Virus	Copy Number	Single-plex	7-plex	5-plex	2-plex	Single-plex	7-plex	5-plex	2-plex
FMDV Asia 1 PAK	47204	+	+	+		+	+	+	
	4720	+	+	+		+	-	+	
	472	+	+	+		-	-	-	
	47	S	-	S		-	-	-	
	4	-	-	-		-	-	-	
SVDV ITL 19/99	2333	+	+	+		+	+	+	
	233	+	+	+		+	-	+	
	23	+	S	+		-	-	-	
	2	S	-	S		-	-	-	
	0.2	-	-	-		-	-	-	
CSFV Alfort187	7031	+	+	+		+	+	+	
	703	+	+	+		+	-	+	
	70	+	-	+		-	-	-	
	7	S	-	S		-	-	-	
	0.7	-	-	-		-	-	-	
VESV-Cal	1305	+	+	+		+	+	+	
	130	+	+	+		+	-	+	
	13	+	+	+		-	-	-	
	1	-	-	-		-	-	-	
	0.1	-	-	-		-	-	-	
ASFV Lisbon 61 [a]	300	+	+	+		+	+	+	
	30	+	+	+		+	+	+	
	3	+	+	+		-	-	-	
	0.3	S	-	S		-	-	-	
	0.03	-	-	-		-	-	-	
PCV2 B [a]	800	+	+		+	+	+		+
	80	+	+		+	+	+		+
	8	+	+		+	-	-		-
	0.8	S	-		S	-	-		-
	0.08	-	-		-	-	-		-
PRRSV LV	222	+	+		+	+	+		+
	22	+	+		+	+	S		+
	2	+	+		+	-	-		-
	0.2	S	-		S	-	-		-
	0.02	-	-		-	-	-		-

\+ Positive detection
\- No detection
S Suspect call
[a] DNA plasmid material

2.6.0. Figure Legend:

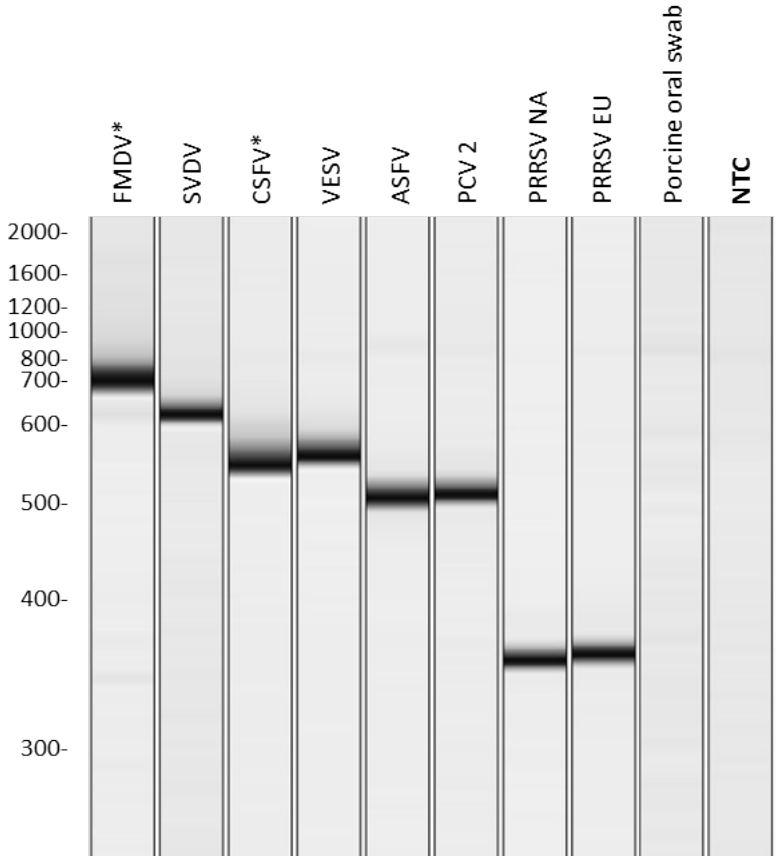


Figure 2.1. QIAxcel gel image of amplified products after multiplex RT-PCR of representative strains of the seven targeted swine viruses. Nucleic acid extracted from oral swabs taken from healthy pigs were used to access the specificity of the PCR. Asterisk represents the use of *in vitro* transcribed RNA, NTC: no template control. QIAxcel 50-3000 bp ladder was used in the program for analysis and 300-2000 bp markers are shown.

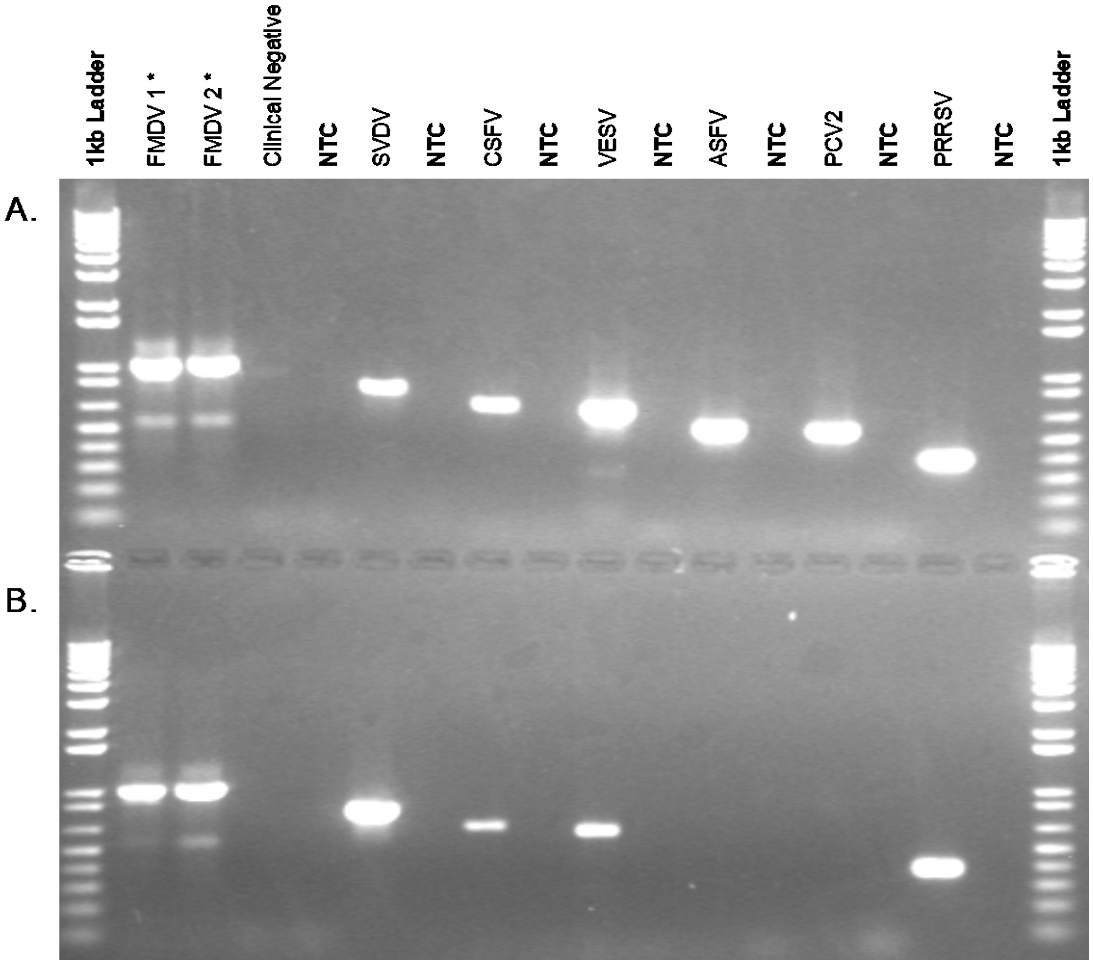


Figure 2.2. 1% agarose gel of seven target swine viruses in single-plex with unmodified primers (A) and TriLink CleanAmp™ primers (B). All single-plex reactions run with a no template control (NTC). Clinical negative sample extracted from swab material from a healthy pig. Ladder used was 1kb plus (Invitrogen, Burlington, ON). Asterisk represents transcribed RNA template.

A.
1+ KB Ladder
2000
1650
1000
850
650
500
400
300
200
A
Iran 1/96 (1)
Arg 2/2001 (2)
Arg /86 (3)
COL /85 (4)
Iran /99 (5)
Iraq 24/64 (6)
Cruzeiro/Bra/55 (7)
O
Manisa (8)
TAW 10/97 (9)
UKG 11/2011 (10)
BFS /860 (11)
C
Noville (12)
Resende (13)
Asia
Pak 1/54 (14)
Stamir (15)
SAT1
KEN 4/98 (16)
BOT 1/68 (17)
SAT2
ZIM 5/81 (18)
SWA 1/69 (19)
SAU 1/2000 (20)
ZIM 10/91 (21)
SAT3
BEC 1/65 (22)
ZIM 4/81 (23)
NTC
B.
SVDV
GRE 1/79 (1)
FRA 1/73 (2)
HKN 1/80 (3)
HKN 3/89 (4)
ITL 1/66 (5)
ITL 1/97 (6)
JAP 1/74 (7)
NET 3/92 (8)
Port 1/2003 (9)
SWI 1/74 (10)
UK 27/72 (11)
ITL 19/92 (12)
CSFV
Alfort187 (39)
Brescia (40)
VR1416?? (22)
NL B64 (29)
Yi 3295 (14)
Diepholz (16)
Conge.Trem. (31)
3.2syn (11)
3.3syn (12)
Kanagawa (23)
VESV
Cal
ASFV
Lisbon-61
Lille
Georgia
PCV2
PCV2 B
2.5
PRRSV
Vaccine 2.5
1.4 (1-3)
MLV
vaccine 2.5
LV (EU)
93 44927
NTC #1
1200
1000
800
700
600
500
400
300
200
100
C.
2000
1600
1200
1000
800
700
600
500
400
300
200
100
267
270
271
272
273
274
277
278
279
281
PRRSV Piglet Negative
PRRSV VNL
267
270
271
272
273
274
277
278
279
281
PRRSV Piglet Negative
NTC
Internal Control
D.
2000
1600
1200
1000
800
700
600
500
400
300
200
100
SIV
PRCV
TGEV
PCV1
VSV
S.suis
Pmultocida
S.ent.Cholerasuis
M.hyopneumonia
BVDV
BDV
PRRSV VNL
SIV
PRCV
TGEV
PCV1
VSV
S.suis
Pmultocida
S.ent.Cholerasuis
M.hyopneumonia
BVDV
BDV
NTC
Internal Control

Figure 2.3. Gel electrophoresis images depicting validation RT-PCR of the seven-plex multiplex against a panel of 80 lab strains. (A) SYBR Safe agarose gel image of 23 FMDV strains including at least two strains per the seven serotypes. (B) QIAxcel gel images depicting validation RT-PCR of 12 SVDV, 10 CSFV, 1 VESV, 3 ASFV, 1 PCV 2 and 8 PRRSV strains, (C) 11 clinical negative oral and nasal swab material with a spiked PRRSV YNL internal control and (D) 11 non-target swine virus and bacteria spiked with PRRSV YNL as an internal control. PRRSV YNL run as an internal control to test that the RT-PCR is working in non-specific samples. QIAxcel 50-3000 bp ladder was used in the program for analysis and 300-2000 bp markers are shown.

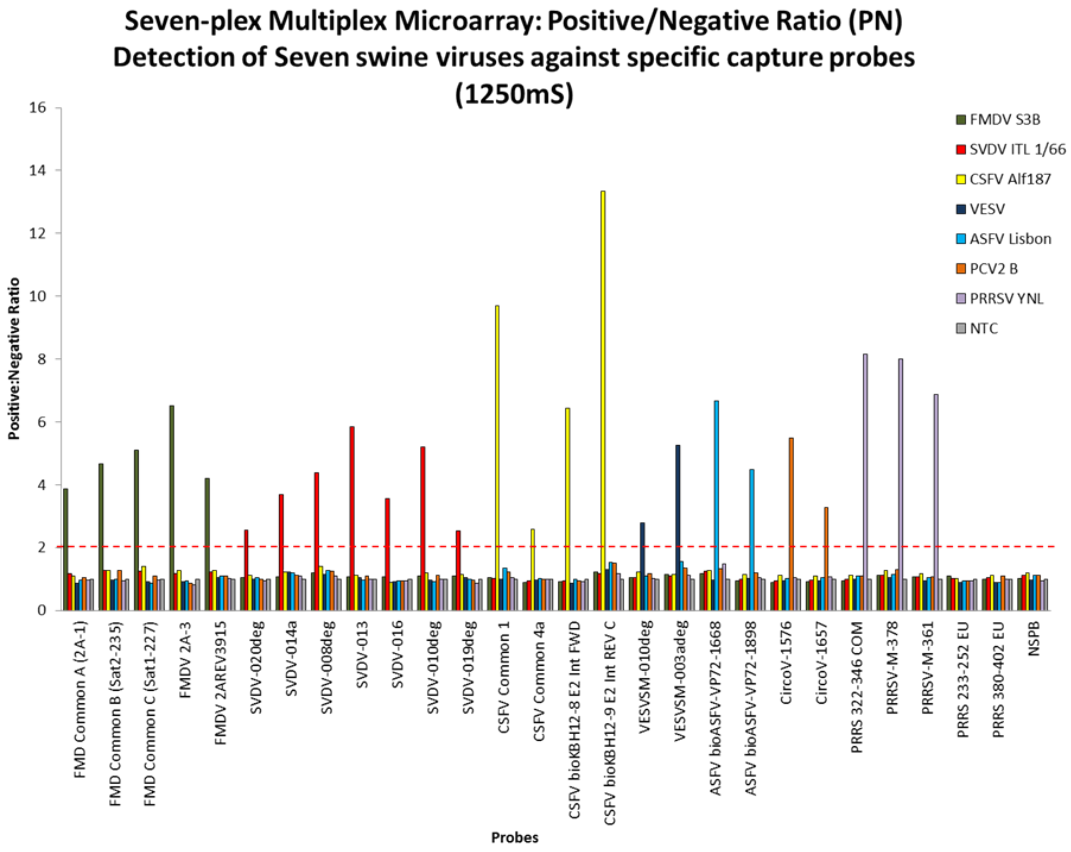
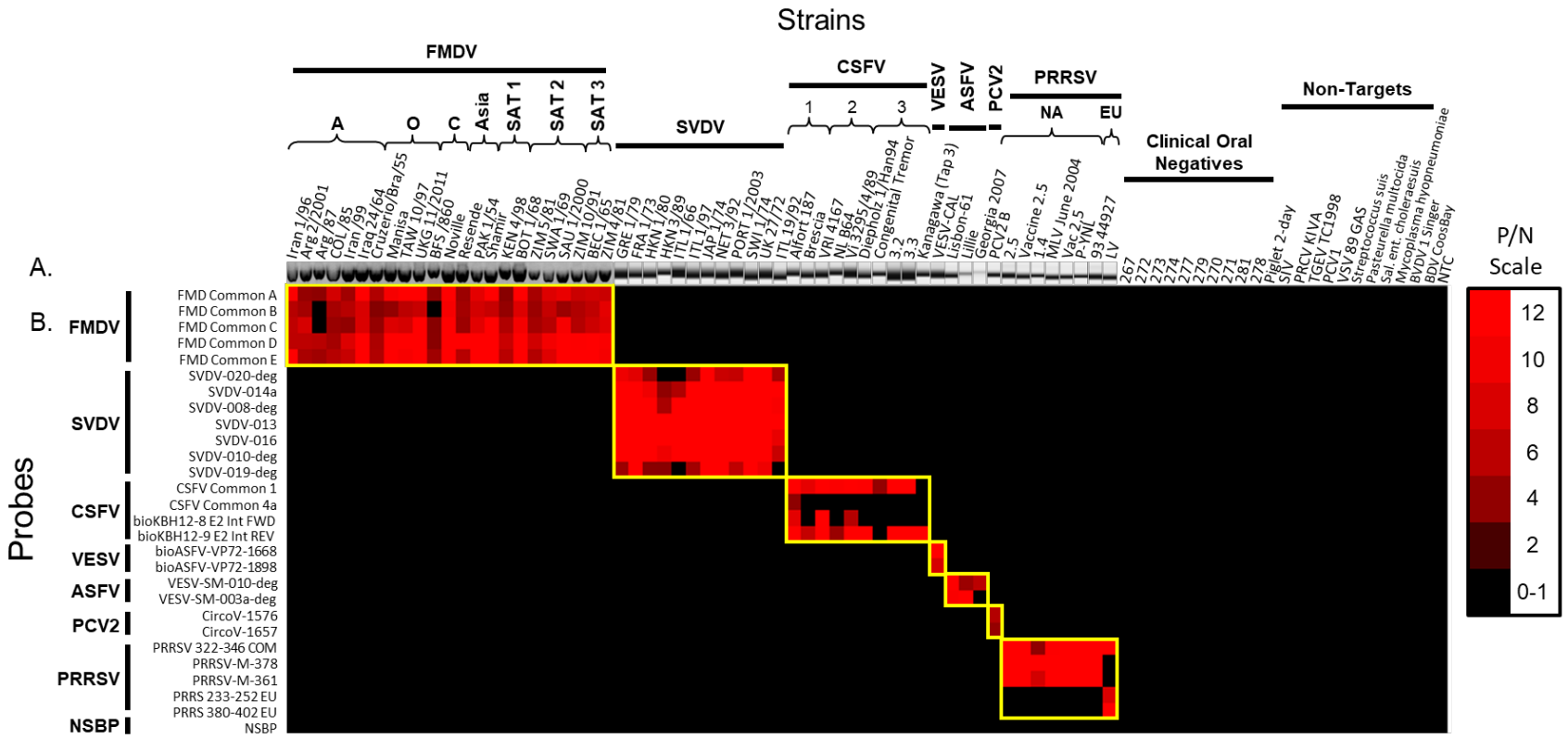


Figure 2.4. Microarray bar graph showing RT-PCR products hybridized to specific capture probes utilizing the optimized NanoChip 400 protocol. Bars represent the ratio of positive fluorescent signal to negative (PN) or background signal from the NTC. Red line shows the PN cut off of 2, marking anything above the line as detected.


Strains
FMDV
A
O
C
Asia
SAT 1
SAT 2
SAT 3
SVDV
CSFV
1
2
3
VESV
ASFV
PCV2
PRRSV
NA
EU
Clinical Oral Negatives
Non-Targets
Iran 1/96
Arg 2/2001
Arg 87
COL/85
Iran/99
Iraq 24/64
Cruzeiro/Bra/55
Manisa
TAW 10/97
UKG 11/2011
BFS/860
Noville
Resende
PAK 1/54
Shamir
KEN 4/98
BOT 1/68
ZIM 5/81
SWA 1/69
SAU 1/2000
ZIM 10/91
BEC 1/65
ZIM 4/81
GRE 1/79
FRA 1/73
HKN 1/80
HKN 3/89
ITL 1/66
ITL 1/97
JAP 1/74
NET 3/92
PORT 1/2003
SWI 1/74
UK 2/72
ITL 19/92
Alfort 187
Brescia
VRI 4167
NL 864
NL 3295/4/89
Diepholz 1/Han94
Congenital Tremor
3.2
3.3
Kanagawa (Tap 3)
VESV-CAL
Lisbon-61
Lillie
Georgia 2007
PCV2 B
2.5
Vaccine 2.5
1.4
MLV June 2004
Vac 2.5
P-YNL
93 44927
LV
267
272
273
274
277
279
270
271
281
278
Piglet 2-day
SIV
PRCV KIVA
TGEV TC1998
PCV1
VSV 89 GAS
Streptococcus suis
Pasteurella multocida
Sal. ent. choleraesuis
Mycoplasma hyopneumoniae
BVDV 1 Singer
BDV Coosbay
NTC
A.
B.
Probes
FMDV
FMD Common A
FMD Common B
FMD Common C
FMD Common D
FMD Common E
SVDV
SVDV-020-deg
SVDV-014a
SVDV-008-deg
SVDV-013
SVDV-016
SVDV-010-deg
SVDV-019-deg
CSFV
CSFV Common 1
CSFV Common 4a
bioKBH12-8 E2 Int FWD
bioKBH12-9 E2 Int REV
VESV
bioASFV-VP72-1668
bioASFV-VP72-1898
ASFV
VESV-SM-010-deg
VESV-SM-003a-deg
PCV2
CircoV-1576
CircoV-1657
PRRSV
PRRSV 322-346 COM
PRRSV-M-378
PRRSV-M-361
PRRS 233-252 EU
PRRS 380-402 EU
NSBP
NSBP
P/N Scale
12
10
8
6
4
2
0-1

Figures 2.5. Seven-plex RT-PCR amplified viruses visualized using agarose gel and QIAxcel system (A.) and heat map depicting the reactivity of samples against 27 virus-specific capture probes and one non-specific binding probe (NSBP) (B.). The panel of samples includes 58 strains of the seven targeted swine viruses, 11 oral swab samples taken from healthy pigs and 11 non-target virus and bacteria that are associated with livestock. The viral strains tested include representatives of each of the seven FMDV serotypes, three CSFV genotypes, two ASFV genotypes and two genotype linages of PRRSV, genotype 1 & 2. Scale legend shows positive signal in red that represents a positive to negative ratio (PN) of ≥ 2, while negative results in black represent any PN < 2.

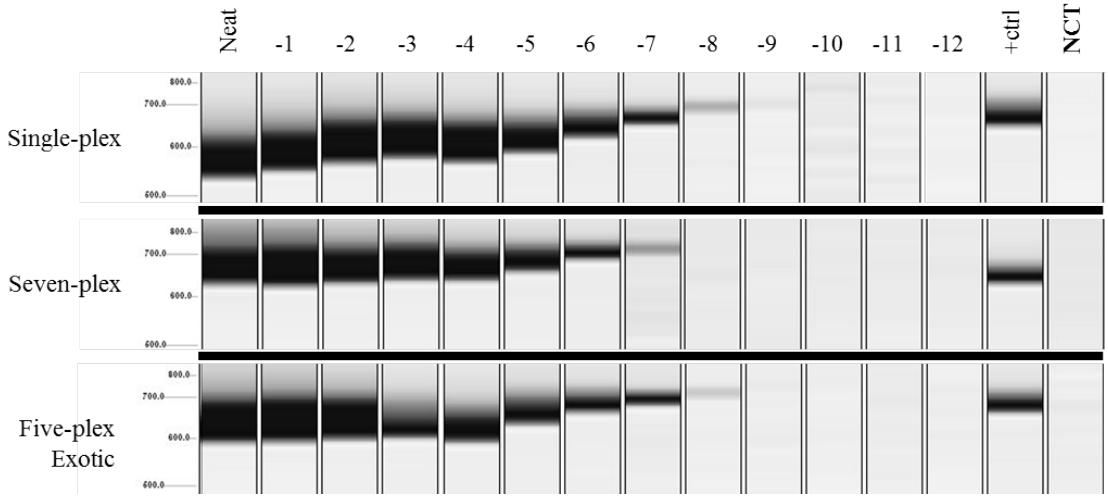


Figure 2.6. QIAxcel gel image of the amplification of SVDV ITL 19/99 10-fold serially diluted transcribed RNA by three RT-PCR tests: single-plex, seven-plex and five-plex (exotic). The three different RT-PCR tests show the effect of additional primers on amplification sensitivity. The positive control (+ctrl) used is a 1/50 dilution of SVDV ITL 19/99 RNA. NTC refers to a no template control. QIAxcel 50-3000 bp ladder was used in the program for analysis and 600-800 bp markers are shown.

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Chapter 3. Integration of High Consequence Swine Virus Assay onto a Portable Sample-to-Answer Automated Detection System

3.1.0. Introduction

In todays market, there is a need for more rapid and sensitive diagnostic testing for detecting pathogens causing foreign animal diseases (FAD). Introduction of FAD causing pathogens into a naïve ecosystem has the potential to cause devastating economic impacts, such as the 2001 foot-and-mouth disease (FMD) outbreak in the UK which resulted in $13 billion US in losses (Thompson et al. 2002) and the classic swine fever virus (CSFV) outbreak in the Netherlands in 1997-1998 that caused roughly $2 billion US in economic losses (Terpstra and de Smit 2000). Outbreaks like this are why organizations such as the World Organization of Animal Health, also known as the Office International des Epizooties (OIE), support the creation of internationally accepted control and prevention methods for highly contagious FADs (Pasick and Kahn 2014).

Some major FAD viruses being targeted for control and prevention are: foot-and-mouth disease virus (FMDV), swine vesicular disease virus (SVDV), vesicular exanthema of swine virus (VESV), CSFV and African swine fever virus (ASFV). These five FAD viruses are selected for control due to their highly infectious nature and because the diseases they cause are clinically indistinguishable signs from one another. An example of this is seen in the vesicular lesions produced by FMDV, SVDV and VESV. Vesicular lesions on the feet and mouth of all cloven hoofed animals are the main signs of all three viruses, yet because FMDV is the most infectious virus known, it is the priority for all diagnostic tests during an outbreak (Banér et al. 2007; Fernández et al.

2008; Lung et al. 2011). However, due to similar disease signs, diagnosis can be difficult. Other confounding pathogens that occur in Canada are porcine circovirus Type II (PCV 2) and porcine reproductive and respiratory syndrome virus (PRRSV). This uncertainty in visual signs can result in reduced response times to implement quarantine procedures to isolate the outbreak, possibly allowing the pathogen to spread. Thus, a rapid and fully integrated and automated detection system is desirable for the simultaneous and accurate differentiation of all target pathogens.

Current gold standard diagnostic detection tests for these pathogens include; virus isolation (VI), enzyme-linked immunosorbent assay (ELISA), virus neutralization and real time polymerase chain reaction (qPCR) (Table 3.1). These tests have been implemented due to their sensitivity and ability to diagnose diseases specifically. The problem with gold standard testing is that tests such as VI are labour intensive and require experienced technicians to complete and they require specific samples or cell lines to grow certain pathogens (Wu et al. 2013). Virus isolation is highly specific at diagnosing diseases but requires at least four days and specific cell lines to propagate the viruses (Yamazaki et al. 2013). ELISAs also have limitations in what samples can be run, including clotted blood and milk which cannot be used (S. Reid et al. 2003).

Point-of-Care (POC) systems represent a novel solution to these issues by reducing manual hands-on time, transfer of material to centralized laboratories and wait times for results. Point-of-Care testing is the use of diagnostic tests that can be performed in close proximity to the patient using easily transported devices (Holland and Kiechle 2005; S. Park et al. 2011). A POC system can combines multiple tests and devices (ie. Extraction, thermocycler and detection instruments) and bridges the gap between them by

offering an integrated instrument. Examples of such as combinations are the reverse transcription (RT) polymerase chain reaction (PCR) and capillary electrophoresis microchips (Kaigala et al. 2008), as well as the integrated sample extraction, PCR and detection microarray of the Rheonix CARD® (Spizz et al. 2012, 2015). The Clinical Laboratory Improvement Amendments of 1988 (CLIA'88) provide the guidelines for POC standards, as well as describe that for an assay to be a POC it must be classified as a "waived" or simple enough for untrained users to use without causing errors. Current nucleic acid testing methods have been classified as "highly complex" which means that tests such as RT-PCR or qPCR assays must be run in a laboratory by trained personnel for accurate results (Holland and Kiechle 2005; Niemz et al. 2011). Assays such as the POCKIT™, an insulated isothermal PCR (iiPCR), have been described in the detection of pathogens such as FMDV and CSFV. The described assay from Ambagala et al. (2016) and Lung et al. (2015) use the basic positive/negative results of the POCKIT™, an economic and portable instrument, to accurately detect FMDV (Ambagala et al. 2016) and CSFV (Lung et al. 2015) from clinical material. With platforms such as these, diagnosis can be made without having time consuming workflows and complex machinery. Having the ability to move the assay from the bench top to the onsite location of the suspected infection will help to mitigate possible contamination from over-handling and sample degradation.

Nexogen Inc. (San Diego, CA) has created a prototype POC sample-to-answer system that incorporates together magnetic bead-based nucleic acid extraction, reverse transcription PCR (RT-PCR) and carbon based microarray detection into a single contained unit. This medical diagnostics machine (MDx) utilizes a single-use cartridge,

along with a fluorescent capturing system for visualization and automated systems. The system is contained within a portable container and utilizes a computer script, run off an accompanying laptop.

The goal of this project is to transfer the seven-plex multiplex assay from Erickson et al. (2017) to Nexogen's integrated and portable MDx system. Testing is split into 3 sections: Proof-of-concept, partial integration- Phase I and Phase II, and Phase III, full integration (Figure 3.1).

3.2.0. Materials and Methods

3.2.1. Samples

For the transfer of the NanoChip 400 assay from Erickson et al. (2017), the same seven swine viruses (Table 3.2) were selected for testing in this study. A panel of eight viral strains was used for initial testing of the MDx assay components: FMDV SAT 3 BEC 1/65, SVDV ITL 19/92, CSFV Alfort187, ASFV Lisbon 61, VESV Cal, PCV2 B, PRRSV YNL and PRRSV LV. Two lineages of PRRSV, North American (NA) and European (EU), were included to validate the ability of the assay to type and differentiate the two virus lineages. Initial testing was done with 1/50 dilutions of extracted viral RNA and DNA from available lab samples, as well as in house generated *in vitro* transcribed RNA for FMDV and CSFV. Most virus work was carried out in the level 3 biosafety laboratories at the Canadian Food Inspection Agency (CFIA) National Centre for Foreign Animal Disease (NCFAD) in Winnipeg, due to the biocontainment requirements for the

five exotic viruses, while work with the indigenous viruses was completed in the CFIA National Centre for Animal Disease (NCAD) Lethbridge Laboratory.

A synthetic construct for FMDV SAT3 BEC 1-65 was made using the 981 base pair (bp) region encoding VP3/VP1/2A/2B portions of the polyprotein and used as a surrogate of viral RNA in assay development due to the absence of FMD viral RNA in Lethbridge. *In vitro* transcribed RNA was generated from plasmids cloned with DNA from FMDV synthetic constructs and CSFV plasmid containing the PCR target gene. For *in vitro* transcription of RNA, the MEGAscript® kit (Life Technologies – Invitrogen, Burlington, ON) was used. A reaction mix was made up for the *in vitro* transcription containing; 1 µg of plasmid, 2 µL of each 75 mM dNTP (ATP, GTP, CTP and UTP), 2 µL of 10x reaction buffer, 2 µL enzyme mix and up to 7 µL nuclease-free water for a final volume of 20 µL. The *in vitro* transcription reaction continued for 4 hours at 37 °C. Template DNA was removed by digestion with 3 µL TURBO® DNase (2U/ µL, Life Technologies-Ambion, Burlington, ON) at 37 °C for 30 min. The transcribed RNA was purified using the RNeasy mini kit "RNA clean-up" protocol (Qiagen, Toronto, ON) as per the manufacturer's specifications. Complete DNA removal was confirmed by PCR amplification of the digested material using a RT-PCR reaction with and without the RT component.

The PCR conditions for the "with RT" reaction were as follows; A 50 µL RT-PCR reaction, run with the SuperScript® III OneStep RT-PCR with Platinum Taq kit, consisted of 1 µL each of 1µM FMDV, CSFV and PCV2 primers (n=6) and 0.5 µL each of 0.5µM SVDV, VESV, ASFV and PRRSV primers (n=8), 25 µL of 2x RT buffer mix, 2 µL of SSIII enzyme mix, 15 µL of ultra-pure water and 1 µL of template. The RT-PCR

reaction was processed on the Veriti thermal cycler (Life Technologies-Applied Biosystems, Burlington, ON) using the following conditions: 55°C for 15 min for RT, then 94°C for 2 min for initial denaturation, followed by 35 cycles of: denaturation at 94°C for 30 min, annealing at 50°C for 1 min and extension at 68°C for 1 min followed by a 5 min final extension step at 68 °C.

The "without RT reactions" were processed the same as the "with RT reactions: with the following differences: amplification was preformed using Platinum® Taq DNA polymerase High Fidelity (Life Technologies - Invitrogen, Burlington, ON), 5 µL of 10x high fidelity buffer, 1 µL of dNTPs (10mM), 50mM $MgSO_4$ 0.2 µL of Platinum® Taq polymerase (5U/ µL), up to 50 µL of ultra-pure water and 1 µL of template RNA. The Veriti thermocycler conditions were the same as the "with RT" reaction, but with no RT step. RNA quantification was done using the Qubit® 2.0 Fluorometer system and RNA broad range kit (Life Technologies-Invitrogen, Burlington, ON). Copy number analysis was done using the following equation:

$$\frac{concentration(ng)\ x\ 6.022x10^{23}/mole}{base\ pair(bp)x\ 1x10^{9}\ {}^{ng}/_{g}\ x\ Molecular\ Weight\ bp\ {}^{g}/_{mole}}$$

3.2.2.0. MDx Sub-Component testing:

Sub-component testing was done as proof-of-concept, showing the individual components ability to perform. All proof-of-concept tests were done on benchtop by hand using the same reagents and template as would be used in the MDx system. Alongside the benchtop work, a control PCR was run to help compare to the results taken from the extracted material (Figure 3.1A).

3.2.2.1. MDx Nucleic Acid Extraction Sub-component

To test the nucleic acid extraction sub-component, serial dilutions of virus stocks were made to test in the MDx cartridge extraction chemistry. PRRSV YNL passage 3 virus stock was diluted to 1/100 in Phosphate Buffered Solution (PBS) pH of 7.5.

For all virus cultures, nucleic acid extractions were done using a proprietary magnetic bead based extraction kit from Nexogen Inc. on the benchtop. Briefly, 200 µL of virus sample was added into 600 µL of magnetic bead containing Lysis buffer, vortexed for 20 s then incubated at room temperature for 1 min and centrifuged briefly. Beads were then collected using a magnet for 1 min after which supernatant was discarded. Beads were washed with 600 µL Wash buffer I (WBI), vortexed for 10 s and centrifuged briefly. Magnetic collection of the beads was repeated, after which the beads were washed with 600 µL of Wash buffer II (WBII). The solution was mixed gently and extracted similarly to WBI. Finally, the beads were resuspended in 50 µL of elution

buffer, vortexed for 10 s, centrifuged briefly, beads were collected by a magnet and the supernatant was collected and saved for testing on RT-PCR.

3.2.2.2. Seven-plex RT-PCR Sub-component

The seven-plex RT-PCR assay from Erickson et al. (2017) was transferred to the MDx RT-PCR tube for testing of the RT-PCR sub-component. Based on machine specifications, the 22 primer (Table 3.3) assay was modified from a 50 µL reaction to 150µL to account for the addition of approximately 50 µL of elution material from the nucleic acid extraction sub-component of the MDx cartridge. Bench top testing of the RT-PCR assay used the following reagents: a 50 µL reaction with 0.25 µL each of 1µM final concentration of FMDV, CSFV and PCV2 primers (n=6) and 0.125 µL each of 0.5 µM final SVDV, VESV, ASFV and PRRSV primers (n=8) all at a starting concentration of 200 µM, 25 µL of 2x RT buffer mix, 2 µL of SSIII enzyme mix, 19.5 µL of ultra-pure water and 1 µL of target sample. The 50 µL reaction was multiplied by 3 to get the 150 reaction volume used in the MDx cartridge. The RT-PCR reaction was run on the Veriti thermal cycler at the following conditions: 55 °C for 22.5 min for RT, then 94 °C for 2 min for initial denaturation, followed by 35 cycles of: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 68 °C for 1 min and followed by a 5 min final extension at 68 °C. Post-amplification reactions were visualized using one of two methods: The QIAxcel (Qiagen, Toronto, ON) was used for all samples except for all FMDV samples in which agarose gel electrophoresis with SYBR® safe DNA gel stain (Life Technologies-Molecular Probes, Burlington, ON) was used in Winnipeg.

3.2.2.3. Electronic Carbon Detection Array and Statistical Analysis

Proprietary carbon based microarrays with 100 hydrogel pads were tested using the selected capture probes (Table 3.4) transferred from Erickson et al. (2017). The probes were prepared to 10 uM in dH_2O and then diluted to 9.5 uM in low salt buffer (LSB, Nexogen, Inc., San Diego, CA). Probes were spotted manually onto the streptavidin containing hydrogel that covers each of the 100 electrode pads. Briefly, the arrays were washed in LSB and then soaked in LSB for 15 min, washed thoroughly with dH_2O and air dried completely before spotting. Select probes were spotted in triplicate onto the hydrogels, 0.35 µL per spot. Two spotting controls were introduced, fluorescence control and a reporter control. The reporter control is a probe that is sequence specific to the tag sequence use on the red universal reporter (RUR, 5`-flurophore-TGTCAAGCGATATACTGC-3`) used to visualize that hybridization has occurred and that the RUR is annealing properly. The fluorescence control was used to indicate location as it will fluores during imaging. After spotting, the array was left at 4°C for 1 hour in a moist container for probe hybridization. The array was washed with Hisproclin buffer containing: 50 mM Histidine (Sigma-Aldrich, St. Louis, MO) and 0.05% proclin® 300 (Sigma-Aldrich-Supelco, Bellefonte, PA). Arrays were stored at 4°C in Hisproclin until needed.

For capture probe screening, arrays were dried completely and a plastic flow cell and translucent cover was adhered to the array. The array was then attached to a "backend" cartridge designed to simulate the final stages of the full cartridge run. Liquid was pumped manually through a system of tubing and syringe. Amplicons were diluted 3:1, to a final volume of 200 uL, in Cap Down A buffer (CdA, Nexogen, Inc., San Diego,

CA) and delivered to the array by a syringe. The RUR was diluted 1:100 to a final concentration of 0.25 uM, and 200 uL was added to the array and incubated for 7 min at room temperature. Electronic addressing of the reporter probe was performed on the MDx instrument at 1.6 nA for 2 min after which the array was incubated for 5 min. The array was washed with 200 uL of LSB at room temperature and LED lights were used to capture an image of the fluorescing probes using 5, 10, 15 and 20 s exposure time.

Images were analyzed using Array-Pro Analyzer 6.3 (Media Cybernetics, Rockville, MD, US) software, measuring the level of fluorescence and extracting raw intensity values into an Excel spreadsheet. The values for the same probe (n=3) were averaged and standard deviations were determined. The standard deviations, divided by the average fluorescence and multiplied by 100%, were used to calculate the percent coefficient of variation (%CV). The %CV is being used to determine any samples than needed to be repeated. The %CV was determined for each probe and when values reached above 50-60% CV, the experiment was repeated. Final data was represented as a positive:negative ratio (PN) where all averaged probe values were divided by the average of the non-specific binding probe (NSBP).

3.2.3.0. Integration of MDx Assay Sub-components:

Integration of the sub-components was done in three phases: I, II, III. Phase I combines the nucleic acid extraction and RT-PCR steps while phase II combines the RT-PCR and microarray components as seen in figure 3.1B and 3.1C respectively. These phases are done using the MDx instrument and cartridge controlled by the computer

scripts (Figure 3.2), but with hands on manipulation of the cartridge where needed. For example, stopping the run and fixing any errors as they occur or to modify the script based on visual observations. The final phase, III, is the full integration of all three components with little to no manual manipulation of the cartridge or the computer script (Figure 3.1D).

3.2.3.1. Partial Integration Phase I: Nucleic acid extraction and RT-PCR

Using a computer script supplied by Nexogen, with slight in-house modifications, complete prototype MDx cartridges were used to integrate the nucleic acid extraction procedure and RT-PCR step. A 150 µL RT-PCR reaction was set up and placed into the MDx PCR tube and attached to the cartridge at position C (Figure 3.2). A standard 50 µL reaction was set up and spiked with previously extracted DNA/RNA and placed on the Veriti thermocycler as a control. With a needle and syringe (Figure 3.2A) 300 µL of virus, diluted in 1/3 in PBS, was injected into the lysis chamber and sealed with hot glue. After the cartridge was inserted into the MDx instrument the computer script was initiated to start the automated nucleic acid extraction procedure where the sample is moved through each chamber of the MDx cartridge in succession (Figure 3.2B). At the end of the extraction, approximately 50 µL of eluted material is automatically transferred to the attached PCR tube, and added to the 150 µL of the seven-plex RT-PCR reaction mixture. The PCR reaction is carried out similarly to section 2.2.2. and after the run is completed the MDx and Veriti control amplicons were analyzed with the QIAxcel to compare the band sizes and intensities.

3.2.3.2. Partial Integration Phase II: RT-PCR and Carbon Detection Array

A Nexogen computer script modified in-house was used to integrate the RT-PCR and microarray sub-components of the process in the cartridge. A 150 µL MDx RT-PCR reaction, spiked with 3 µL of extracted target DNA/RNA, was set up, loaded into the MDx PCR tube (Figure 3.2C) and placed into the MDx instrument. The MDx script ran the RT-PCR stage and after the PCR finished, the amplicon was automatically mixed with 300 µL of Sample Buffer A (SBA) and loaded onto the microarray (Figure 3.2D). Amplicons were electronically addressed to the carbon array at 1.6 nA for 2 min, paused for 5 min and hybridized to 500 µL of RUR reporter probe for 7 min. The array was then washed four times with 400 µL of LSB with an image taken after each wash using exposure times of: 5, 10, 15, and 20 s.

3.2.3.3. Phase III: Full Integration of MDx Seven-plex Assay

The full MDx computer script was used to integrate the entire three assay sub-components: DNA/RNA extraction, RT-PCR and detection microarray. Integration of the three components was done similarly to the phase I partial integration, but removing the QIAxcel check immediately preceding to the RT-PCR and detection steps. The integration is done without any manual manipulation of either the script or cartridge.

3.3.0. Results

3.3.1. Assay Sub-component, Proof-of-Concept Testing

A seven-plex RT-PCR amplicon-to-answer assay was transferred from the NanoChip 400 electronic microarray platform, to the prototype fully automated and integrated “sample-to-answer” MDx platform. The MDx assay was broken down into three subcomponents and evaluated separately before integrating the components together. A panel of the seven target viruses was used to test the three assay components: nucleic acid extraction, RT-PCR and detection microarray. For testing of the nucleic acid extraction sub-component, the nucleic acid extracting chemistry kit supplied by Nexogen was used. Nucleic acid extraction of all 7 target viruses was successfully extracted offline on the bench top, off the MDx instrument, from cell culture material and amplified using the standard RT-PCR from Erickson et al. (2017) (Figure 3.3). For testing of the PCR/RT-PCR sub-component, total nucleic acid from the target viruses were amplified using the MDx thermocycling module in a MDx cartridge on a MDx instrument (Figure 3.4). Resulting amplicons were compared to control amplicons generated using the Erickson et al. (2017) assay, and showed similar amplification (data not shown). To test the microarray detection sub-component, amplicons generated from the offline Veriti thermocycler were applied to the carbon array and run through the hybridization and washing protocols on a MDx cartridge, where all seven amplicons bound to their specific probes (Figure 3.5).

3.3.2. Partial Integration

After demonstrating and establishing proof-of-concept of the MDx sub-component parts, the assay was moved into the partial integration step, Phase I. Phase I of the integration included integration of nucleic acid extraction and RT-PCR on the microfluidic cartridge. Nucleic acid were extracted from lab grown viruses and amplified by RT-PCR using an MDx cartridge and amplicons were visualized on QIAxcel. Amplicons from all seven viruses were detected indicating successful nucleic acid extraction and RT-PCR amplification (Figure 3.6). Phase II integration of RT-PCR with microarray detection demonstrated that using pre-extracted nucleic acid, RT-PCR can be completed and amplicons can be detected by capture probes on the microfluidic cartridge automatically. Four out of seven target virus were detected by their respective probes: 6/7 probes for SVDV, 2/2 for VESV, 1/2 for PCV 2 and 3/5 for PRRSV YNL (Figure 3.7). Three of the seven targets; FMDV, CSFV and ASFV failed to be detected.

3.3.3. Full Integration

A total of 300 uL of laboratory amplified virus were diluted 1:3 in PBS for testing of the fully integrated assay. All target viruses were run from beginning to end using the automated computer program, without any manual manipulations. Four of the seven targets, SVDV, VESV, PCV 2 and PRRSV YNL and LV, were run from beginning to end and detected by their specific probes. The four targets were successfully extracted, amplified by RT-PCR (Figure 3.8a) and detected on their respective capture probes

(Figure 3.8b) fully automated on the microfluidic cartridge. FMDV, CSFV and ASFV failed to amplify after extraction, resulting in no detection.

3.4.0. Discussion

The objective of this project was to transfer a previously developed and optimized assay (Erickson et al. 2017) to a new prototype, fully automated and integrated "sample-to-answer" microarray detection platform.

Transfer of the assay was accomplished by first testing the individual assay sub-components that make up the "sample-to-answer" system: Nucleic acid extraction, RT-PCR and detection by carbon microarray. Each sub-component was tested with the seven target viruses, as well as the European serotype for PRRSV (Table 3.2). The extraction chemistry kits supplied by Nexogen Inc. (San Diego, USA) contained all of the reagents required for extracting nucleic acid from virus samples on the MDx microfluidic cartridge. Though offline testing was done to mimic the nucleic acid extraction on the MDx cartridge, there were differences between the offline and on-cartridge processes. For example, samples were vortexed rigorously on a stand alone vortex instrument to ensure proper mixing of sample and reagents offline, while the cartridge uses a syringe to mix the reaction components 10 times. Though both samples are mixed, the more vigorous mixing offline may aid in cell lysis and washing, allowing for better nucleic acid extraction and result in a stronger signal to noise ratio than the automated cartridge system. Similarly, testing of the carbon microarray offline manually allowed for greater control of the movement of liquids. Thus, if pressure is lost or a leak was observed due to

quality of the prototype cartridge, more liquid can be immediately introduced manually to compensate. Therefore the results show a successful transfer and proof that the individual assay components are compatible with the target viruses and the prototype MDx.

In the process of fully automating the assay, the individual sub-components were systematically integrated together. Phase I (integration of nucleic acid extraction and RT-PCR) worked well for all the targets, but phase II (integration of RT-PCR and microarray detection) had issues with detection of three targets. Four of the targets viruses were successfully detected automatically demonstrating the integrated system works. However, manufacturing quality control issues (ie. leaks) and coding errors with computer coding contributed to the failure of the FMDV, CSFV and ASFV targets being amplified by RT-PCR and detected. In many runs, little to no amplification was seen after spiking the MDx PCR tube with template DNA/RNA as input material, despite the positive controls ran successfully offline in a thermocycler (data not shown). The temperature of the MDx RT-PCR reaction can be monitored on a peltier interface graph showing Time vs. Temperature. Issues with failure to reach set temperatures or took too long time to reach temperature is frequently observed (data not shown). This is the most likely reason for the weak or no amplification after RT-PCR that was observed.

Fully automated and integrated detection was achieved for four of the seven viruses, SVDV, VESV, PCV 2 and PRRSV. During full integration, extraction efficiencies of FMDV and CSFV in cell culture media were low, most likely due to low sample titre. However, one experiment showed that ASFV was successfully extracted and amplified by PCR on the MDx cartridge, but was not detected due to a manufacturers defect in the microfluidics going onto the microarray of the cartridge (data not shown).

Amplicon size may have attributed to FMDV and CSFV failing to amplify after extraction. FMDV and CSFV are two of the larger amplicons in the assay at around 980 and 650 bp respectively. Though, with SVDV reacting strongly, low titre of the available lab virus may be the reason for the failed detection.

Replication of the results was a major issue during the move to the MDx. The MDx is a second generation prototype instrument and during the development process multiple instrument and cartridge errors occurred. About 66 full cartridges were used to generate results, however only a portion of them yielded usable results. Due to these inconsistences a true comparison between the NanoChip 400 and the MDx was not completed. Though four of the seven viruses were successfully run on the fully integrated MDx, the assay couldn't be truly validated due to the missing viruses and the low reproducibility of the system. More work on the MDx would be needed to address these issues.

Fully automated and integrated sample-to-answer pathogen detection is a powerful concept for rapid POC diagnostics with the potential application in hospitals, clinics or farms. Since outbreaks can happen suddenly and spread fast, such as the 2001 UK FMDV outbreak (Thompson et al. 2002), it is imperative that veterinarians and inspectors have the ability to rapidly diagnose and implement quarantine zones around infected herds to contain the spread of the pathogen. Nexogen's advanced prototype MDx machine represents a novel way of filling this niche. Its fully integrated design and portable instrumentation makes it a potentially useful tool that can be used for on-site detection. Other types of POC devices partially integrated the full workflow. For example, the JANUS automated workstation incorporates sample extraction to qPCR

(Vandemeulebroucke et al. 2010). PCR to detection POC devices can be a simple as a lab-on-chip mounted to a self-made electronics board (Tan et al. 2014) or the VereFlu™ lab-on-chip system that incorporates a standalone PCR/microarray instrument and reader system (Teo et al. 2011). The MDx instrument goes beyond these other instruments by integrating together sample extraction, RT-PCR and microarray detection to create a simple user-friendly diagnostic tool that technicians can use with minimal training.

Future directions for this project include further validation of this POC technology. The advanced MDx prototype instrument and cartridge used in this project is undergoing further refinement to improve manufacturing reproducibility and fix computer coding errors. As part of the optimization undertaken in this project, utilizing higher RT temperatures and by altering the computer scripts manually to allow longer incubations or mixing steps improved results. Nexogen has also announced the development of new systems such as the Nexi-Dx, a card based sample-to-answer system or the CAS-100, a three cartridge sample-to-answer system that uses the same cartridge system as the MDx (http://dhodko5.wixsite.com/mysite). Systems such as these are the future for the POC industry.

To conclude, the seven-plex multiplex assay from Chapter 2 was successfully transferred from the NanoChip 400 electronic microarray to Nexogen's prototype fully integrated and portable MDx instrument. Proof-of-concept was achieved by successfully extracting, amplifying and detecting a seven viruses on each of the instruments individual sub-components as well as for partial integration. Full, hands off, integration of the seven-plex assay was demonstrated for four of the seven viruses, including two lineages for PRRSV (NA and EU).

3.5.0. Table Legend

Table 3.1. List of gold standard tests available for target virus detection

Virus	Virus Isolation	PCR/ TaqMan®	Virus Neutralization	ELISA	Other
Foot-and-Mouth Disease virus [a]	✓	✓	✓	✓	Complement Fixation
Swine Vesicular Disease virus [b]	✓	✓	✓	✓	N/A
Classical Swine Fever virus [c]	✓	✓	N/A	✓	Neutralizing peroxidase-linked assay (NPLA)
African Swine Fever virus [d]	✓	✓	N/A	✓	Indirect fluorescent antibody test (IFAT)
Vesicular Exanthema of Swine virus *	✓	✓	N/A	✓	Complement Fixation
Porcine Circovirus Type II**	N/A	✓	N/A	N/A	In situ Hybridization
Porcine Reproductive and Respiratory Syndrome virus [e]	✓	✓	✓	✓	Immunofluorescence assay

N/A Not applicable

✓ Test available

✓ Test prescribed by OIE

OIE – World Organization for Animal Health 2018: 2.1.8 [a], 2.8.8 [b], 2.8.3 [c], 2.8.1 [d], 2.8.6 [e]

Zimmerman, et al. 2012: 493*, 412-413**

Table 3.2. High consequence viruses targeted in this study

Virus	Abbreviation	Family	Genome Type	Gene Target	Clinical Signs
Foot-and-Mouth Disease	FMDV	*Picornaviridae*	+ssRNA	VP3/VP1/2A/2B	vesicular lesions [a]
Swine Vesicular Disease	SVDV	*Picornaviridae*	+ssRNA	3C/3D	vesicular lesions [b]
Classical Swine Fever	CSFV	*Flaviviridae*	+ssRNA	E1/E2	hemorrhages, immunosuppression, lesions [c]
African Swine Fever	ASFV	*Asfarviridae*	dsDNA	VP72	hemorrhages, lesions [d]
Vesicular Exanthema of Swine	VESV	*Caliciviridae*	+ssRNA	Polymerase	vesicular lesions [e]
Porcine Circovirus Type II	PCV2	*Circoviridae*	ssDNA	Capsid	immunosuppression, lesions [f]
Porcine Reproductive and Respiratory Syndrome	PRRSV	*Arteriviridae*	+ssRNA	Matrix	reproductive failure, respiratory dysfunction, lesions [g]

Zimmerman et al. 2012 pgs 597[a], 604[b], 541[c], 399[d], 494[e], 410[f], 468[g]

Table 3.3. List of primers used for multiplex amplification of seven high consequence viruses

Virus	Genomic Region	Primer	Sequence (5`-3`)	Amplicon size (bp)	Reference
FMDV	VP3/VP1/2A/2B	VP3com980(1)	GCT GAT TAC GCG TAC AC	971	Erickson et al. 2017
		VP3com980(2)	GCT GAC TAC GCG TAC AC		Erickson et al. 2017
		VP3com980(3)	GCG GAT TAC GCC TAC AC		Erickson et al. 2017
		VP3com980(4)	GCG GAT TAC GCG TAC AC		Erickson et al. 2017
		VP3com980(5)	GCA GAT TAC GCG TAT AC		Erickson et al. 2017
		VP3com980(6)	GCA GAC TTT GCA TAC AC		Erickson et al. 2017
		VP3com980(7)	AGT GAC TTC TCC TAC AC		Erickson et al. 2017
		VP3com980(8)	GCT GAC TAT GCT TAC AC		Erickson et al. 2017
		VP3com980(9)	GCA GAC TTT GCC TAY AC		Erickson et al. 2017
		FMDV 2B Rev 4026-S-Deg2*	GCG GAC ACC ARC CGG TTR AAG TC		Erickson et al. 2017
SVDV	3C/3D	SVDVCV-3C-17a-F-(5875bp)	CAG CGG CAC TCC TCA GAC ACT AC	791	Lung et al. 2011
		SVDVCV-3D-3a-R-(6642bp)*	GAG TTT CAG GCA CGT AAA CCA CAC		Lung et al. 2011
CSFV	E1/E2	KBH12-5 E1 Ext FWD	AGR CCA GAC TGG TGG CCN TAY GA	671	Paton et al. 2000
		KBH12-6 E2 Ext REV*	TTY ACC ACT TCT GTT CTC A		Paton et al. 2000
ASFV	VP72	King Long - Fwd Primer	ATA GGA TTA AAA CCT ACC TGG AAC ATC TCC G	537	King et al. 2003
		King Long - Rev Primer*	GGT ACT GTA ACG CAG CAC AGC TGA ACC GTT CTG		King et al. 2003
VESV	Polymerase	VESVSM-2-F-(5101bp)	CGA CTC GAT GGA CCT GTT CAC ATA CG	649	Lung et al. 2011
		VESVSM-5-R-(5749bp)*	CGT AGA GGT CGG TTA GGT CCT TTC TG		Lung et al. 2011
PCV 2	Capsid	CircoV-1222F	GTA ATC AAT AGT GGA ATC TAG GAC	534	Erickson et al. 2017
		CircoV -1760R*	TTC GTT TTC AGA TAT GAC GTA TC		Erickson et al. 2017
PRRSV	Matrix	PRRS-Mtrx- F2	AAG GTA AGT CGC GGC CGA C	379	Erickson et al. 2017
		PRRS-Mtrx -R2*	TGC CRC CCA ACA CGA GGC		Erickson et al. 2017

* Reverse primers contain a complimentary tag sequence for Red Universal Reporter probe at the 5` end (Huang et al., 2009).

Table 3.4. List of Capture probes used in this study for printing on MDx carbon microarray.

Virus	Genomic region	Probe name	Sequence (5`biotinylation* - 3`)	Reference
FMDV	VP3/VP1/2A/2B	1. FMD Common A	AAG TTG GCN GGA GAC GTB GAG TCC AAC CC	Erickson et al. 2017
		2. FMD Common B	AAC TTY GAC CTG TTA AAG TTG GCB GGA GAC GTT GAG TC	Erickson et al. 2017
		3. FMD Common C	AAC TTC GAC CTG TTA AAG TTG GCY GGA GAC GTT GAG TCC AAC CCT	Erickson et al. 2017
		4. FMD Common D	GAG TCC AAC CCT GGG CCY TTC TTC TTC	Erickson et al. 2017
		5. FMD Common E	GAG AYG TBG AGT CCA ACC CTG GGC CYT T	Erickson et al. 2017
SVDV	3C-3D	6. SVDV-020-deg	AAG AGA CAT YCT ATC CAA GAA GAC CAG AGA CCT TRC CA	Lung et al. 2011
		7. SVDV-014a	GGG TAG CGC CGT TGG GTG TGA CC	Lung et al. 2011
		8. SVDV-008-deg	GTG GCY YTG GGT ATC AAG AAA AGA GAC AT	Lung et al. 2011
		9. SVDV-013	GCA ATG AGG CAG ACA TTT GGA AAC CTA TA	Lung et al. 2011
		10. SVDV-016	AAA GAG ACA TCC TAT CCA AGA AGA CCA GAG ACC T	Lung et al. 2011
		11. SVDV-010-deg	TAT GGT CTA AAC YTR CCA ATG GTA ACC TA	Lung et al. 2011
		12. SVDV-019-deg	ACA ACT AGC CAC ACT RGA CAT YAG CAC KGA RC	Lung et al. 2011
CSFV	E1/E2	13. CSFV Common 1	CTT AAK GTG GTY AGT AGG AGG TAY	Erickson et al. 2017
		14. CSFV Common 4a	CTG RAY GAC GGR ACY GTY AR	Erickson et al. 2017
		15. bioKBH12-8 E2 Int FWD	TCR WCA ACC AAY GAG ATA GGG	Paton et al. 2000
		16. bioKBH12-9 E2 Int REV	GAT GAC TTY GGR TTY GGR CTG TG	Paton et al. 2000
VESV	Polymerase	17. VESV-SM-010-deg	CCA CYA TGG CTA CTA CTC AYA CGC TTC TGT CGT TTG AC	Lung et al. 2011
		18. VESV-SM-003a-deg	CGG ATG CTG ARA TAA CGC CTA TCC C	Lung et al. 2011
ASFV	VP72	19. bioASFV-VP72-1668	CTG CTC ATG GTA TCA ATC TTA TCG A	King et al. 2003
		20. bioASFV-VP72-1898	ACG GCY GAT CTT GTG GTA TC	King et al. 2003
PCV 2	Capsid	21. CircoV-1576	ATA TCC GAA GGT GCG GGA T	Erickson et al. 2017
		22. CircoV-1657	GAC GAG CCA GGG GCG GCG GC	Erickson et al. 2017
PRRSV	Matrix	23. PRRS 322-346 COM	TAC ATT CTG GCC CCT GCC CAT CAC G	Erickson et al. 2017
		24. PRRSV-M-378	GGC AAA TGA TAA CCA CGC ATT TG	Erickson et al. 2017
		25. PRRSV-M-361	GGC TTT CAT CCG ATT GCG GCA AAT G	Erickson et al. 2017
		26. PRRS 233-252 EU	TTG TCA CCC TTC TGT GGG GC	Erickson et al. 2017
		27. PRRS 380-402 EU	CGT CTG GTA ACC GAG CAT ACG CT	Erickson et al. 2017
Controls		28. NSBP 4328	CAA AGT GGG AGA CGT CGT TG	Hindson et al. 2008
		Reporter control	GCA GTA TAT CGC TTG ACA AGT AGA AAC AAG GGT GTT TT	Modified from Lung et al. 2012

* Probes modified with 5` biotinylation for binding to streptavidin pad on NanoChip 400 microarray (Takahashi, et al. 2008)

3.6.0. Figure Legend

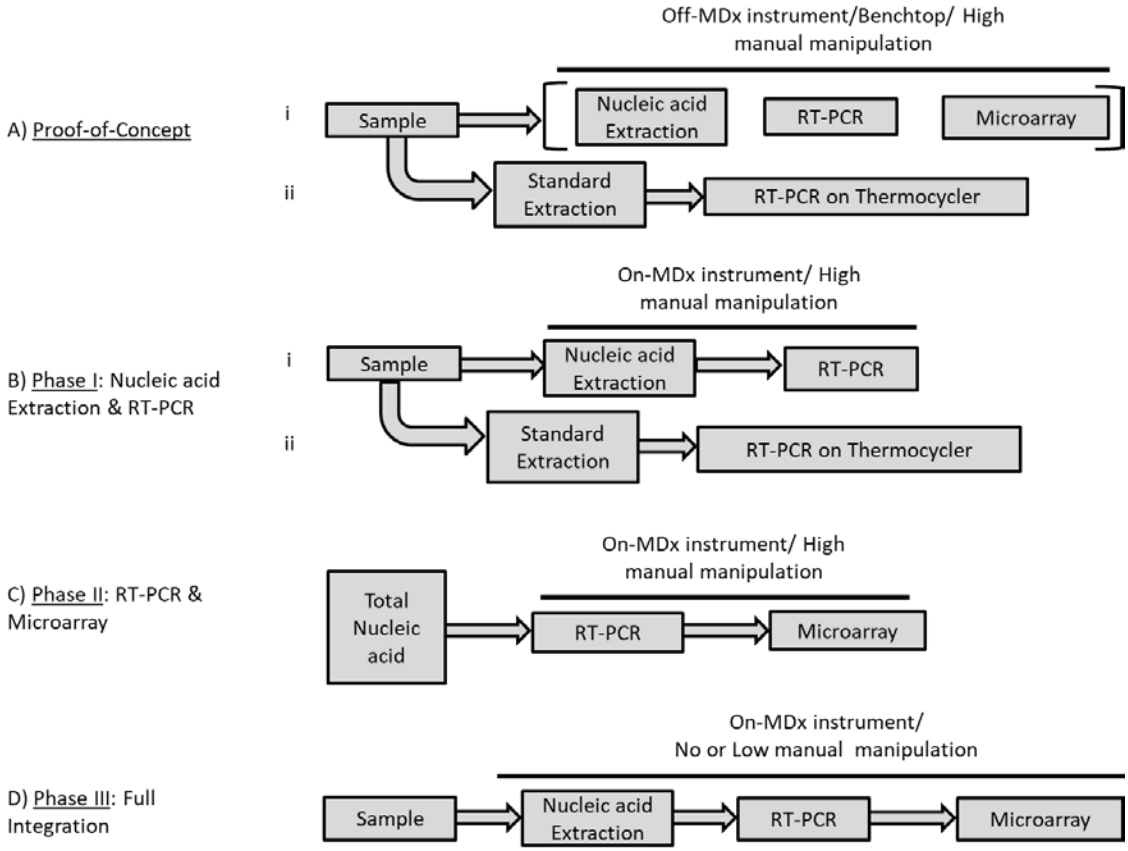

Figure 3.1. Work flow chart illustrating the phases of development for the MDx detection assay. Samples are run manually on the benchtop using the same chemistry/conditions and microfluidic cartridge as would be run on the MDx instrument (i). A control PCR, using total nucleic acid extracted using Qiagen kit, was run along side the MDx PCR on a thermocycler (ii) for comparison (A). The extraction and RT-PCR components were integrated and tested on the MDx instrument using a MDx cartridge (i). A control RT-PCR was run along side on a Veriti Thermocycler (ii) for comparison (B). The RT-PCR and microarray components were integrated using previously extracted nucleic acid, with amplification and subsequent detection run on the MDx instrument (C).

All components were integrated together and samples run on the MDx instrument from nucleic acid extraction to detection with very little or no manual handling of the cartridge (D).

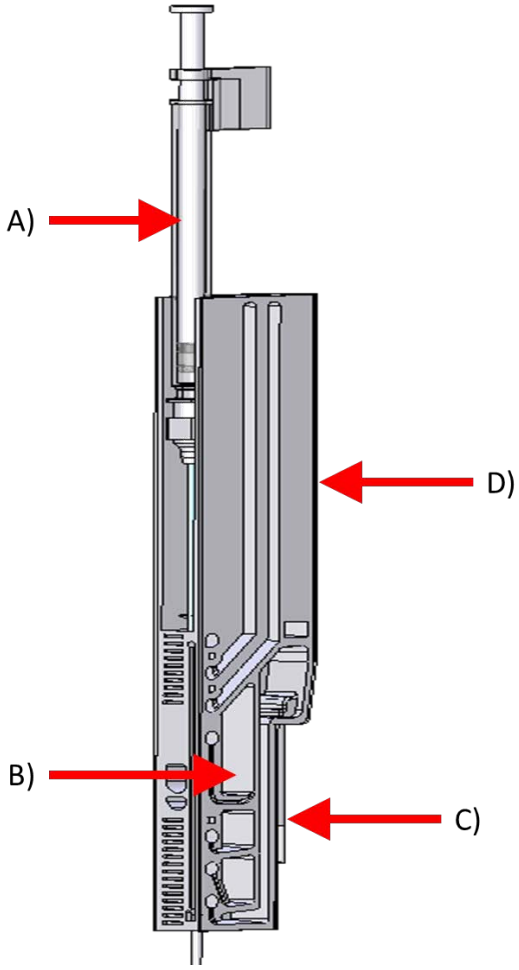

Figure 3.2. Schematic diagram of the prototype MDx cartridge for sample-to-answer detection. A sample injection needle (A) is used to allow the MDx instrument to transfer the sample into the nucleic acid extraction chamber (B). After nucleic acid extraction is complete, the eluted product is transferred using microfluidic channels to the PCR tube (C), that is inserted into the MDx thermocouple, where the extracted total nucleic acid is amplified. Amplicons are then transferred via microfluidic channels to the carbon microarray (D) where amplicons hybridize to immobilized capture probes and are detected using reporter oligonucleotides probes tagged with fluorophores. Schematic supplied by Nexogen Inc. (San Diego, California).

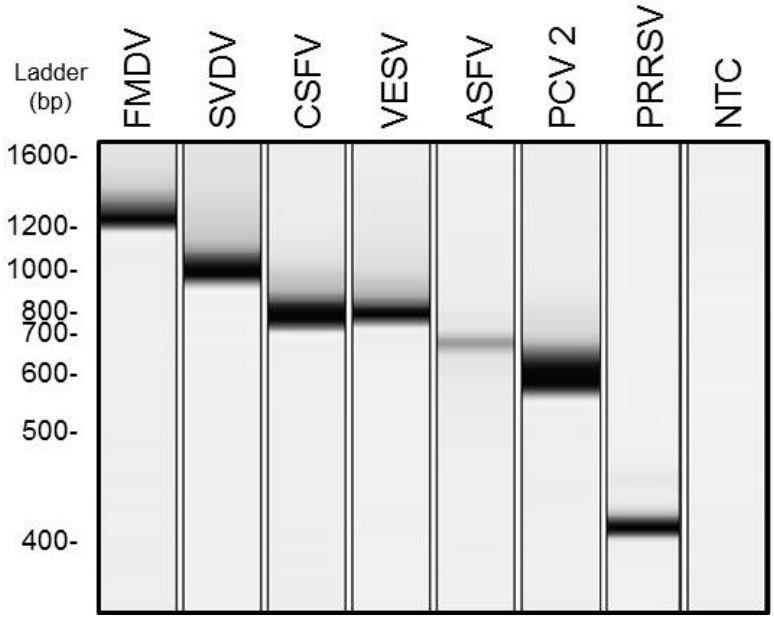

Figure 3.3. QIAxcel gel image of amplification of swine virus targets extracted using MDx extraction chemistry on the benchtop manually and amplified using standard RT-PCR on a thermocycler. Lab propagated virus were used for extraction and amplification. No template control (NTC) was included as a negative control. QIAxcel 50-3000 bp ladder was used in the program for analysis and 400-1600 bp markers are shown.

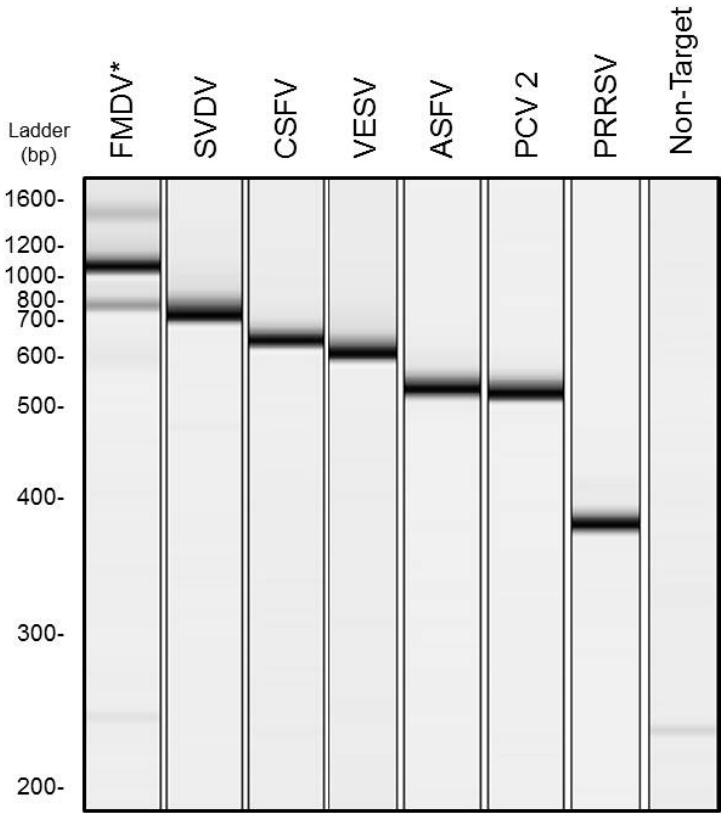

Figure 3.4. QIAxcel gel image of proof-of-concept amplification of swine virus targets using thermocycling module on the MDx cartridge. Standard extracted material were used for amplification. Non-target BVDV RNA were included to demonstrate assay specificity. QIAxcel 50-3000 bp ladder was used in the program for analysis and 200-1600 bp markers are shown. Asterisk represents the use of transcribed RNA as template material.

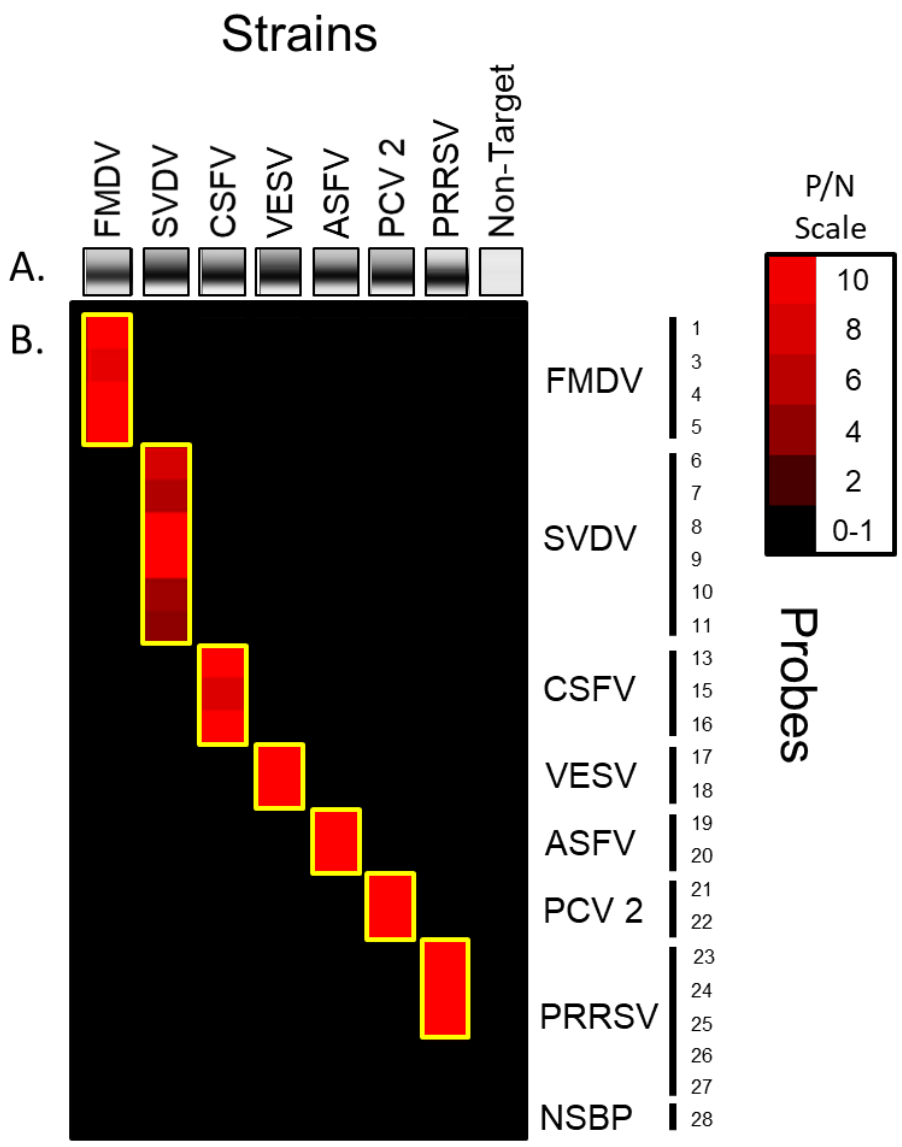

Figure 3.5. Proof-of-concept testing of RT-PCR amplicons on the MDx cartridge carbon-based microarray. (A) QIAxcel gel image and (B) microarray heatmap of offline generated amplicon (Veriti thermocycler) run on a MDx cartridge. Nucleic acid extracted from lab propagated virus was used in the RT-PCR. Scale legend shows positive to negative (PN) ratios. Positive ratios of ≥ 2 are represented in red, while negative ratios

shown in black are a PN ratio of < 2. Non-specific binding probe (NSBP) used as a negative control probe.

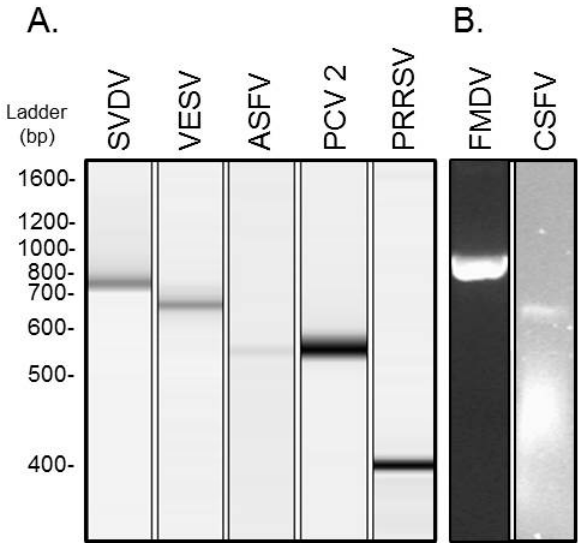

Figure 3.6. Gel images of phase I partial integration of the nucleic acid extraction and RT-PCR. (A) QIAxcel gel image and (B) SYBR Safe Agarose gel RT-PCR generated amplicons. Lab propagated viruses extracted and amplified automatically using the MDx cartridge. Amplicons generated with no manual manipulation of the MDx instrument. QIAxcel 50-3000 bp and SYBR Safe 1kb plus ladder were used. The 400-1600 bp markers are shown.

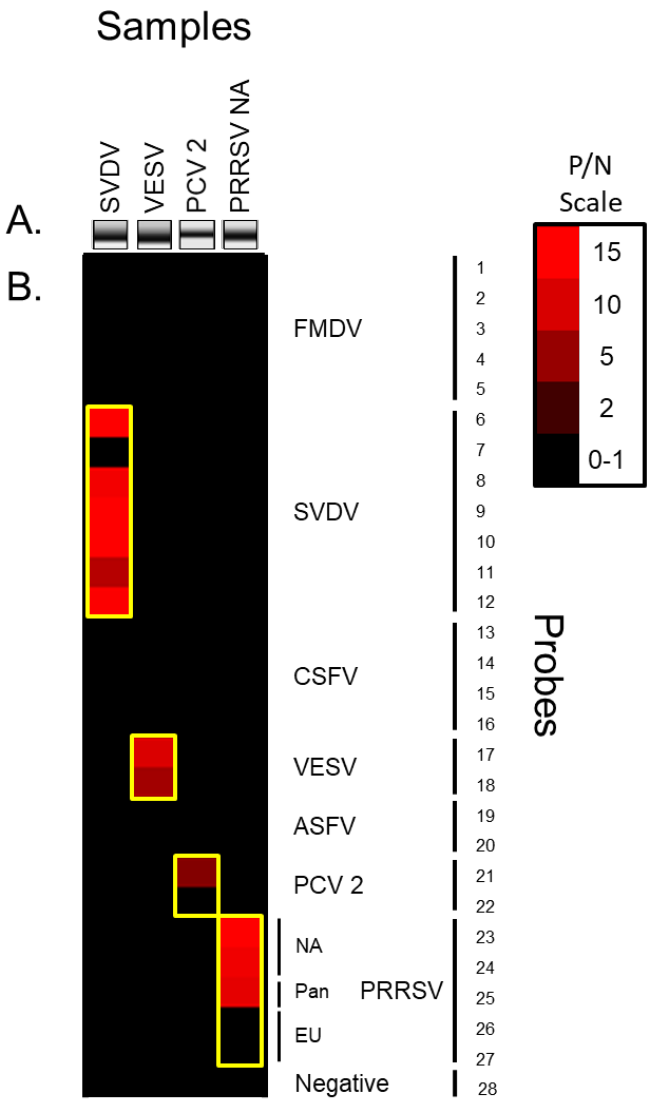

Figure 3.7. Phase II partial integration of MDx RT-PCR and microarray detection sub-components using MDx instrument and cartridge. QIAxcel gel image (A) and microarray heatmap (B) of phase II partial integration. Nucleic acid extracted off instrument from lab propagated virus were used to produce amplicons Scale legend shows positive to negative (PN) ratios. Positive ratios of ≥ 2 are represented in red, while negative ratios shown in black are a PN ratio of < 2. Non-specific binding probe (NSBP) is used to determine the background signal from non-specific binding.

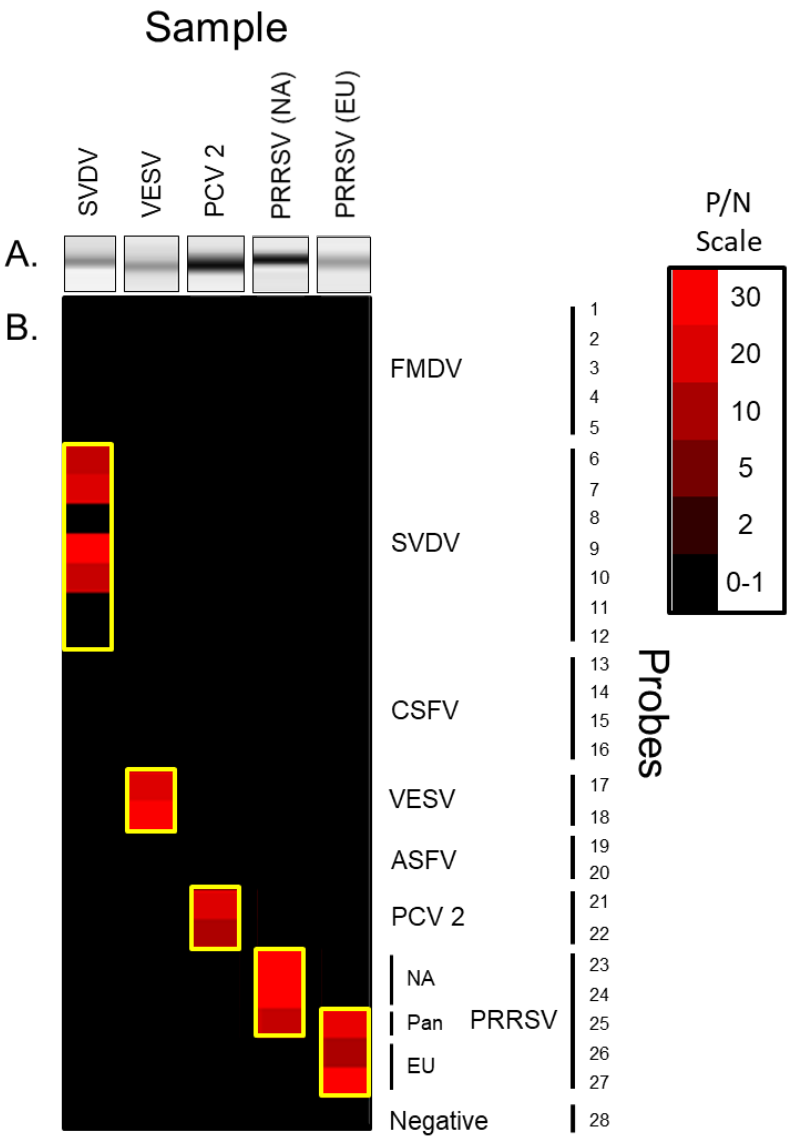

Figure 3.8. Phase III full integration of all MDx sub-components. QIAxcel gel image (A) and microarray heatmap (B) of full integration of the MDx. Lab propagated virus material was used as input into the MDx cartridges. All samples were extracted and detected on the microarray with no manual manipulation of the computer script or cartridge. Scale legend shows positive to negative (PN) ratios. Positive ratios of ≥ 2 are represented in red, while negative ratios shown in black are a PN ratio of < 2. Non-

specific binding probe (NSBP) is used to determine the background signal from non-specific binding.

3.7.0. References

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Chapter 4. Conclusions and Future Directions

4.1.0. Conclusions

The objective of this thesis was to develop, optimize and then transfer a multiplex RT-PCR and microarray assay on the NanoChip 400 electronic microarray to the new MDx system. Using multiplex RT-PCR technology, a seven-plex multiplex RT-PCR assay was developed, validated and incorporated into a corresponding NanoChip 400 assay. It was shown that all samples tested (n=58) amplified and reacted to their appropriate capture probe while negative clinical samples (n=11) and non-target (n=11) samples were un-reactive. The NanoChip 400 assay was then successfully transferred to a proto-type fully integrated and portable, MDx, electronic microarray system. The MDx was successful in automatically performing nucleic acid extraction, RT-PCR amplification and detecting four of the seven high consequence viruses with no manual manipulations of the machine or cartridge.

These two microarray systems, the NanoChip 400 and MDx, represent novel user-friendly detection platforms. Their ease of use simplifies user training needed to conduct multiplex diagnostic tests in both the laboratory and in the point of need. “Point-of-care” (POC) instruments are desirable as it allows detection on-site, eliminating the need for transport of samples.

Many examples exist where delayed diagnosis has caused greater damage, as in the case of the foot-and-mouth disease (FMD) outbreak in 2001 for the UK (Thompson et al. 2002). FMD is the most contagious animal virus known and can spread very quickly,

affecting thousands of animals in a matter of days. After the outbreak was identified and contained about 6.5 million animals, infected or not, were culled to halt the spread of the virus. The UK suffered an estimated $13 billion loss to its economy (Thompson et al. 2002). Another example of a viral disease slipping through surveillance was the 1997 outbreak of classical swine fever (CSF) in the Netherlands. This outbreak affected around 1.1 million pigs, which were culled to prevent spread of the disease causing pathogen (Terpstra and de Smit 2000). In more recent news, the OIE reported in their annual reports that there has been an outbreaks of African swine fever virus (ASFV) in Europe, Africa and China. They report that as of March 28, there have been a loss of nearly 3000 pigs, approximately 97% being from Asia, due to the ASFV outbreak (http://www.oie.int/fileadmin/Home/esp/Animal_Health_in_the_World/Report_13_Current_situation_of_ASF.pdf). Due to the high risk of pathogen spread, it is imperative that rapid and user-friendly diagnostic tests be developed to reduce the time needed for disease diagnosis.

The NanoChip 400 is a potentially useful addition to a diagnostic laboratory. Its 400 test sites can be independently activated by platinum electrodes to allow negatively charged amplicons to be attracted to test sits for instantaneous hybridization. Thus results are obtained in a few hours compared to the gold standard virus isolation tests that can take a few days to generate results. Although the NanoChip 400 can readily produce results, size makes it more suitable for use in a lab. This as well as the fact that it requires the samples to be processed for nucleic acid extraction and PCR separately, makes it more of a laboratory instrument. The MDx, however, does fall into the category of a POC instrument. Its fully integrated design allows for the user to take it to the site of any

possible outbreak where the instrument can perform the entire process automatically and generate results in under three hours. Being a proto-type instrument, the MDx and its cartridges needs further development before commercialization.

In conclusion, this applied thesis has covered the development, optimization and validation of a seven-plex RT-PCR multiplex assay and corresponding user-friendly electronic DNA microarray system for the detection and differentiation of five exotic and two indigenous swine viruses. The assay was successful at detecting all seven viruses from clinical material and with high sensitivity. The seven-plex assay was then successfully transferred to a fully integrated and portable machine, where it achieved detection of five out of seven viruses under complete automation from extraction to microarray detection.

4.2.0. Future Directions

The seven-plex RT-PCR multiplex and corresponding Nanochip 400 and MDx systems are potentially useful tools in molecular diagnostics of livestock diseases. Once the systems are fully validated and recognized as true diagnostic tests, the assays could be employed as a tool for inspectors in the fight to safeguard our food supply and livestock industry. Both laboratory technicians and veterinarians can be trained to use the technology to rapidly confirm possible viral outbreaks as well as being able to quickly implement quarantine of affected herds to halt the spread of the disease. Another positive attribute of the assays is that it can greatly reduce the number of animals that have to be

culled. It can do this by reducing test times for animals suspected of being infected, thereby allowing for animals that would have been killed otherwise in a blanket attempt to halt the spread of the disease.

Future directions for this project are a two fold answer. One avenue is to do further optimizations on the system in an attempt to increase the sensitivity. One such optimization is to reduce the amplicon size of the larger targets such as FMDV, which sits at 981 bp. Doing this could aid in amplification of multiple targets simultaneously, by theoretically allowing the polymerase to amplify target genomic regions with little competition or size biasing. A full validation of multiple infections is needed for this assay. This would require a panel of different combinations of the seven high consequence viruses, from two to all seven. The number of combinations is very large so it would need to be worked down to the most likely combinations, PCV2 and/or PRRSV used as a base infection as they are found in pigs under healthy conditions. With the baseline viruses the combinations would be as follows: vesicular viruses FMDV, SVDV and/or VESV and then the hemorrhagic viruses CSFV and ASFV. The resulting amplicons would then be run on the NanoChip 400 microarray to determine the efficacy of the assay in detecting multiple infections. Along with this, a second validation trial is needed to run more strains of the target viruses. In chapter 2, 58 samples are validated against the multiplex. Of those 58 only three were PRRSV isolates and three more were ASFV. A greater cateloge of isolates is needed to truly validate the assay.

The other future direction for this project would be to continue testing the MDx instrument or moving to a more advanced system. The MDx instrument used in this thesis was a second generation prototype. Being that it was a prototype instrument there

were many mechanical issues that needed to be fixed and worked through. Having a system where the instruments and reagents are reliable is crucial. During the work on the MDx, only a small number of runs were completed due in part to the instruments repair status and the location of the samples needed to run in the assay. The adaptation of the NanoChip 400 assay to the MDx required the work to be done in a BSL-2 laboratory where only transcribed RNA and plamid constructs could be used in place of their BSL-3 viral counterparts. The true virus work for five of the seven viruses had to be carried out in the BSL-3 laboratory in Winnipeg. In the BSL-3 labs, FMDV, SVDV, VESV, CSFV and ASFV viruses were run under high containment protocols, requiring them to be run in a special room where tubes being removed had to be soaked in Virkon S dissinfecant and people were required to shower out. This made any rapid progress difficult and when errors occurred, full days of work would be lost. Compounding the location and instrument issues, work on detecting these viruses requires meticulous planning and execution. More time working on the MDx system is the most important step in developing a reliable assay.

As stated previously, the NanoChip 400s size made it more suitable for use in a laboratory. Recently, SavionDiagnostics (Ashdod, Israel) has developed an updated version of the previous NanoChip 400 instrument, the NanoChip 400 XL. The XL series is a smaller version of its predecessor, including in its design the capability to run PCR on the instrument. The addition of the PCR component allows for a more rapid and streamline processing of diagnostic samples, reducing the need for training on multiple instruments. SavionDiagnostics currently only offers NanoChip 400 kits for human

diseases and genetic mutation identification but they are in the process of adding veterinary diagnostic kits to their repertoire.

The next step for the seven-plex multiplex assay is to incorporate additional new targets and upcoming technologies such as the POCKIT (Balasuriya et al. 2014; Lung et al. 2015; Ambagala et al. 2016), TwistDx (H. Liu et al. 2017; Garrido-Maestu et al. 2018) and the Rheonix system (Spizz et al. 2012, 2015; Z. Chen and Zhu 2016; Z. Chen et al. 2013). The greatest ability of the multiplex assay described is that it can be altered easily to incorporate different targets such as bacteria. Another multiplex assay developed by Lung 2015 is used to identify eight targets of the Porcine Respiratory Disease Complex; four viruses and four bacterium that commonly infect pigs (Lung et al. 2015). Of the upcoming pieces of technology, the isothermal system found both the POCKIT and TwistDx represent a breakthrough in the development of POC instrumentation. Isothermal PCR utilizes one constant temperature to amplify gene products either using the insulated isothermal PCR method (iiPCR, POCKIT) or the recombinase PCR amplification (RPA, TwistDx) for detecting a single target (H. Liu et al. 2017; Garrido-Maestu et al. 2018). The iiPCR uses a small tube that is heated at the bottom to around 95 °C. This heating then causes a gradient of 95-60 °C (bottom-top) to form in the liquid and generates a small current that brings the DNA target into the different temperature areas, where it follows the steps of PCR amplification: At the bottom the DNA is denatured, at the top the primers anneal and in the middle it begins extension (Ambagala et al. 2016; Lung et al. 2015). For the TwistDx, the RPA method uses a low temperature, around 37 °C, but utilizes recombinase proteins to help with primer binding and double helix denaturing (Swift et al. 2016). The major advantage to using these instruments is the

speed at which they can generate amplicons. Using one temperature means that there are fewer waiting steps involved where the thermocycler needs to either heat or cool to a specific temperature. The lack of a thermocycler also makes the system less expensive as it only needs a heating block that reaches one temperature, instead of fluctuating between multiple temperatures over time.

The Rheonix Encompass MDx system represents what the Nexogen MDx system could possibly become in the future. The Encompass MDx is a fully automated system that utilizes the Rheonix CARD® (Chemistry and Reagent Device) that is controlled by microfluidics and air pressure. The Rheonix CARD® have already been used in human medicine applications, such as assays for sexually transmitted diseases (Spizz et al. 2012) and a Warfarin genotyping assay (Spizz et al. 2012, 2015) and has already had veterinary applications for livestock pathogens (Lung et al 2018).

In the end, the purpose of these tools is to safeguard our environment and economy. Surveillance is the ultimate goal of projects such as these; creating new procedures and utilizing novel tools or techniques in ways that can detect pathogens faster or at lower titres than before. At the speed at which pathogens mutate and evolve, applied research such as the use of the NanoChip 400 and the Nexogen MDx has become indispensable to the protection of not only our personal health, but also the health of our livestock and economy.

4.3.0. References

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