Development of a Method for the Detection of Aleutian Mink Disease Virus in Water Samples

by

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ABSTRACT

Aleutian mink disease virus (AMDV) causes significant loss to the mink industry in Nova Scotia (NS). Contaminated water is a speculated virus source therefore my objective was to develop a method for detection of AMDV in water samples. Initially, the quality and quantity of AMDV DNA extracted by four commercial kits was evaluated by PCR. Next, viral capture by adsorption-elution filtration was tested using spiked water samples. The 1MDS filter method was superior or comparable to the $MgCl_2$ filter method and outperformed the $AlCl_3$ method. Lastly, 16 watershed samples from Southwest NS and 11 samples from two AMDV-infected ranches were tested. Five ranch samples from the two ranches tested positive. Three samples, containing more than 22 virus copies/µL after concentration were quantifiable. Pre-concentration, these samples contained 16,787 to 33,471 virus copies/mL. In conclusion, although further optimization is required, a method for the detection of AMDV in water samples was developed.

LIST OF ABBREVIATIONS AND SYMBOLS USED

$	dollar
%	percent
°C	degrees Celsius
AE	adsorption-elution
$AlCl_3$	aluminum chloride
AMDV	Aleutian Mink Disease Virus
ANOVA	analysis of variance
AX	Axygen extraction kit
BEV	bovine enterovirus
bp	base pair
CI	confidence interval
Cl^-	chloride ion
cm	centimeter
Ct	cycle threshold
DB	Dynabeads viral extraction kit
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleotide triphosphate
dH_2O	deionized water
DOC	dissolved organic carbon
E	amplification efficiency
EDTA	Ethylene diaminetetraacetic acid
EV	enterovirus
F	farm
FPV	panleukopenia virus
g	gram
GADPH	glyceraldehyde-3-phosphate dehydrogenase
GPS	global positioning system
GuSCN	guanidine thiocyanate
H_2SO_4	Sulfuric Acid
HAdv	human adenoviruses
HEV	human enterovirus
HDPE	high density polyethylene
Hwy	highway
IAC	internal amplification control
kb	kilobase

L	litre
LCN	$\log_{10}$ of viral copy number
LD	$\log_{10}$ of dilution series
LOD	limit of detection
Log_{10}	logarithm based 10
LOQ	limit of quantification
M	molar
min	minute
mg	milligram
$MgCl_2$	magnesium chloride
mL	millilitre
mM	millimole
MVM	minute virus of mice
n	number
ng	nanogram
NA	nucleic acid
nm	nanometer
NS	Nova Scotia
NTU	nephelometric turbidity units
Pan-1	calicivirus
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming unit
PO_4^{3-}	phosphate ion
ppt	part per thousand
QI	Qiagen DNA extraction kit
qPCR	quantitative PCR
RNA	Ribonucleic acid
SAS	Statistical Analysis System
SD	standard deviation
TE	Tris-EDTA
TFF	tangential flow filtration
uS	micro Siemens
USEPA	United States Environmental Protection Agency
UV	ultra violet
V	volt
VFF	vortex flow filtration
vs	versus

w/v	weight by volume
WS	watershed
x g	times gravity
ZR	Zymo Research DNA extraction kit
μL	microlitre
μm	micrometer
X^2	Chi-square

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CHAPTER 1. INTRODUCTION

1.1 ALEUTIAN DISEASE

Aleutian disease (AD) was first identified on a mink farm in the USA, by Hartsough and Gorham (1956) and has since been identified in all mink pelt producing countries. The disease was observed in mink of Aleutian coat color and was originally thought to only affect this color type. In actuality, Aleutian coat color is an autosomal recessive trait and these mink suffer from immune deficiency. Aleutian mink are more vulnerable but mink of any coat color are susceptible to the disease causing virus (Karstad and Pridham, 1962). AD is a viral (Aleutian mink disease virus [AMDV]) induced immune complex disease that results from viral antigen-antibody complexes (Porter et al., 1969). Variations in the DNA sequence have resulted in different viral strains, with varying degrees of pathogenicity (Hadlow et al., 1983) and it is possible for a mink to be infected with more than one strain at any given time (Gottschalck et al., 1991). Depending on the strain of the virus, the genotype and age of the mink, varying degrees of the disease have been reported (Hadlow et al., 1983) and include death (Alexandersen et al., 1989), decreased fertility (Hansen and Lund, 1988) and lower pelt quality (Farid and Ferns, 2011). The origin of the virus, whether ranch or wild, is not known, but transmission from wild animals to ranch animals is a suspected cause of re-infection (Oie et al., 1996). The virus has also been detected in wild animals, including raccoons (Oie et al., 1996), otters (Manas et al., 2001) polecats, stone martens, pine martens, genets (Fournier-Chambrillon et al., 2004) and skunks (Allender et al., 2008).

1.2 AMDV Structure

AMDV is a member of the family *Parvoviridae*, sub family *Parvovirinae*, genus

Amdovirus, and is commonly referred to as a parvovirus. Parvoviruses are very small

and AMDV has a diameter between 24-28 nm (Bloom et al., 1980). The viral DNA is

single stranded and has approximately 4800 base pairs. The DNA is contained within a

non-enveloped icosahedral capsid and codes for 5 proteins; three non-structural and

two capsid forming (Christensen et al., 1993). The capsid is formed by 60 protein

subunits (McKenna et al., 1999) that are arranged to produce a negative surface charge

(Alexandersen, 1990). Specific binding domains are also present on the surface of the

capsid (Dimmock et al., 2007). Similarities exist between AMDV and viruses in the same

family, such as human parvovirus B19, feline panleukopenia virus (FPV), and minute

virus of mice (MVM) (McKenna et al., 1999).

1.3 Importance of Viral Detection in Environmental Water Samples

The presence of viruses in recreational water (coastal water, lakes and rivers) (Jiang et

al., 2001; reviewed in Fong and Lipp, 2005), drinking water (Verheyen et al., 2009) and

irrigation sources posses serious health risks to humans, animals and plants. Many

viruses, including those that cause hepatitis, meningitis, fever, rash, gastrointestinal and

respiratory illness, and hepatic as well as nervous system infections, have been found in

water samples (reviewed in Bosch, 1998; reviewed in Griffin et al., 2003). Animal

viruses, causing diarrhea, reproductive problems, pneumonia, lesions, and neurological

disorders, have also been detected in environmental water samples (Fong and Lipp,

2005). Very few viral copies are required to cause infection, thus the sensitivity of

detection and identification methods are extremely important. Viral contamination

testing is necessary to ensure that water is safe for both human and animal

consumption, as well as for the irrigation of food crops.

1.4 VIRUSES THAT CONTAMINATE THE ENVIRONMENT AND INFECT VIA THE FECAL-ORAL PATHWAY

There are at least 100 different types of infectious human viruses excreted in human

waste that can be transmitted via contaminated water (Berg, 1983; Bosch, 1998). This

group of viruses is known as enteric viruses and includes viral families such as

Picornaviridae, Adenoviridae, Caliciviridae and Reoviridae (reviewed in Griffin et al.,

2003). Although the exact number of enteric viruses that cause infection in livestock

species is not known, it is expected to be as large as those that infect humans.

Parvoviruses, identified in the 1960's, infect both humans and animals, including cats,

dogs, wolves, minks, ferrets, skunks, and raccoons (Barker and Parrish, 2008).

Parvoviruses, including AMDV, have been detected in the feces of infected species and

are transmitted via the fecal-oral route (Alexandersen et al., 1989; reviewed in Steinel et

al., 2001; Barker and Parrish, 2008). Exposure to contaminated environments, including

soil and water, is a suspected cause of parvovirus and specifically AMDV infections

(Addie et al., 1998; Frolich, 2002; Farid et al., 2012). Some farmers believe that ranch

re-infection of AMDV occurs after disturbance to soil during construction (mink

ranchers, personal communication). There is a variety of literature reporting the

detection of viruses in water and environmental sources (Fong and Lipp, 2005; De Paula

et al., 2007; Verheyen et al. 2009; Victoria et al., 2009; Prado et al., 2011). However,

despite the fact that AMDV is suspected to be persistent in the environment and such environments may be a source of infection (Farid et al., 2012), there is apparently no literature on the detection of this virus in environmental samples.

1.5 ROUTES OF VIRAL ENTRY TO WATER SYSTEMS

Common routes of viral entry into water systems include leaking sewage or septic systems, agricultural and urban runoff, sewage output in marine waters (Fong and Lipp, 2005) and inadequate water treatment (Bosch, 1998). Virus particles are excreted in feces of infected humans at very high levels, e.g. 10^5 to 10^{11} hepatitis virus particles/g, 10^{10} rotavirus particles/g and 10^9 norwalk virus particles/g (Bosch, 1998; Griffin et al., 2003; Atmar et al., 2008). Thus, very little fecal volume is needed to cause significant contamination. Just like their human counterparts, animal viruses are present and excreted at high levels in feces. For example, 10^{10} parvovirus/g was reported in canine feces (Filipov et al., 2011). Animal waste can contaminate surrounding water systems; for instance, porcine enteroviruses have been found in surface run off from sites where pig manure has been spread. Run off from cattle feedlots tested positive for bovine enteric viruses when samples were tested using cell culture techniques, after filtration with Millipore filters and elution with beef extract (Derbyshire and Brown, 1978). Bovine enteroviruses have also been detected in river water samples (Fong et al., 2005). Although AMDV has not been previously detected in environmental samples, there is potential for it to be in water systems. The runoff from mink farms has been identified as a possible cause of artificial eutrophication, algae blooms and the decrease in water

quality of lakes and rivers in southwest NS (Taylor, 2009; Werring, 2011; Brylinsky, 2011). It is highly probable that this runoff also contains AMDV.

1.6 SURVIVAL OF VIRUSES AND THEIR INFECTIVITY IN THE ENVIRONMENT

Numerous factors impact the concentration and infectivity of viruses in the environment. Viruses are unable to replicate outside host cells, therefore, once in the environment they are greatly affected by dilution. Enteric viruses remain infectious in the environment for longer periods of time than do bacteria (Bosch, 1998; Fong and Lipp, 2005) and different types of viruses loose infectiousness at different rates. Bacteria are more susceptible than viruses to changes in pH, temperature and ultra violet (UV) light (Sinton et al., 2002). Bacteria may also be affected by nutrient starvation (Bogosian et al., 1996). Although bacteria counts are used to infer water safety, these issues perhaps explain why bacteriological indicators result in an incomplete assessment of water quality (Bosch, 1998).

Temperature is a major factor affecting viral survival. Heat affects viruses by denaturing proteins in the capsid (Wetz et al., 2004) and viral presence persists longer at lower temperatures (Fong and Lipp, 2005). Yates et al. (1985) found temperature to be an important factor of viral decay for poliovirus 1, echovirus 1, and MS-2 coliphage in ground water samples. Results of plaque assays showed that 77.5% of the variation in decay rates could be explained by temperature. In marine virus survival studies, reviewed by Griffin et al. (2003), increased viral detection was reported for samples with lower water temperatures. Furthermore, norovirus concentrations from the Tamagawa

River (Japan) samples were highest for samples collected during the winter months; summer samples had concentrations of 1.1 $\log_{10}$ units lower for NV-G1 and 2.0 $\log_{10}$ units lower for NV-G2 than winter samples (Haramoto et al., 2005). Temperature was the suspected cause although many other factors such as the amount of precipitation or actual animal presence may have contributed. Non-enveloped viruses, including picobirnaviriadae, parvoviruses and circoviruses, are more resistant to high heat than other viruses (Fong and Lipp, 2005). Storage of AMDV for 180 days at 22°C did not reduce antigen titres (Cho and Ingram, 1974), nor did storage at 56°C for 30 minutes (Porter and Cho, 1980), but temperatures of 80°C for 30 minutes did inactivate the virus (Porter and Cho, 1980). The minute virus of mice, another parvovirus, remained infectious after 60 minutes at 80°C (Carter and Saunders, 2007), while the canine parvovirus was still infectious in feces after 6 months at room temperature (Frolich, 2002). Another factor known to affect viral survival is UV light. Both natural and artificial UV irritation damage viral capsids and DNA, thus decreasing virus infectivity (reviewed in Richards, 1999).

Water source, and more specifically characteristics such as suspended solids and their ionic strength, the presence of enzymes and flow rate are factors that may also influence survival rates of viruses in environmental samples (Fong and Lipp, 2005; Fong et al., 2005). It is believed that viral association with suspended particles can provide defense from degrading factors such as UV and enzymes (Fong and Lipp, 2005). Although a stability study of poliovirus found that when filtered and unfiltered seawater were spiked and incubated at 22 °C, the virus survived longer (30 days) in the filtered

sample than in the unfiltered (22 days), as measured by both cell culture and RT-PCR (Wetz et al., 2004). The results indicated that the degrading effects of proteases, nucleases and other enzymes present in the unfiltered sample outweighed the potential protective effects of the suspended solids.

As would be expected, survival rates are also dependant on viral type. Using correlation rates based on cell culture assays over 55 days, poliovirus, hepatitis A virus, and adenovirus type 40 and 41, spiked in tap water and incubated at 4 °C, were predicted to reach 99% inactivation after 41, 56, 92 and 304 days, respectively (Enriquez et al., 1995). As previously mentioned, there is minimal information available on the survival of AMDV in the environment; this type of information would be very useful for further management of the virus.

As discussed above, many factors influence the survival and concentrations of viruses in environmental samples. One publication used a variety of factors to predict viral presence. When stream flow, water temperature, mean rainfall, dissolved oxygen and chlorophyll-a concentration were used as indicators for the presence or absence of human enteroviruses (HEV), bovine enteroviruses (BEV) and human adenoviruses (HAdV), DNA, their presence was correctly predicted 79-85% of the time (Fong et al., 2005). This study did not identify how these factors actually affect virus particles but it does indicate the complex relationship between sample characteristics and viral presence. Fong and Lipp (2005) also identified suspended solids, turbulence and

nutrient content of water as factors that influence the survival of viruses, though no references were given in their review.

1.7 VIRAL DETECTION STRATEGIES

The study of viruses in environmental samples is not novel. In 1940, when sewage samples suspected to contain poliomyelitic virus were inoculated into monkeys, a mortality rate of 60% confirmed viral presence (Paul et al., 1940). Wallis and Melnick (1967) used electronegative filters to concentrate viruses from water and Sobsey and Jones (1979) used electropositive filters. After decades of research for the detection of viruses in water, many different methods have been developed (Hill et al., 1971). Though many different mechanisms and procedures are utilized, all methods include three basic steps: water sampling, viral concentration, and finally viral detection (Fong and Lipp, 2005).

Bacterial indicators are frequently used to measure water quality. Although feces is a source of both bacteria and viruses, measures such as total coliforms are not adequate to infer viral safety as they do not measure, nor do they have a relationship to, the presence of viruses(reviewed in Bosch 1998; Fong and Lipp, 2005). There were no correlations found between total coliforms, fecal coliforms or enterococci and the presence of human adenoviruses (Jiang et al., 2001). This study was conducted using three viral concentration methods, coastal water samples from California and nested PCR. In another study, the presence of fecal coliform and total coliform was not significantly related to the presence of human or bovine enteric viruses in samples

collected from the Altamaha River, Georgia, USA (Fong et al., 2005). These studies

indicate that bacterial load is not an indication of viral load and that bacterial counts

alone are not sufficient to determine water quality. Thus, it is necessary to test samples

for viral contamination. Though many methods have been developed for viral

detection, several factors influence recovery and large variability is reported in viral

yields. While viral detection research has been more heavily concentrated on human

viruses, the concentration methods are not host specific, and should work, or have

worked, as effectively with animal viruses of the same type.

1.7.1 Viral Concentration Methods

Due to dilution effects viruses are typically found in environmental samples in low

concentrations as such, samples must be concentrated prior to detection. The average

diameter of a virus is 20 to 350 nm (Health Canada, 2010). Therefore, they are too small

for effective mechanical, size filtration (Fong and Lipp, 2005). This is particularly

important for AMDV which has a diameter of approximately 26 nm. Other limitations of

size filtration include lack of sensitivity, filter clogging and co-concentration of detection

inhibitors.

Vortex flow filtration (VFF) and tangential flow filtration (TFF) are ultrafiltration methods

used for viral concentration. The VFF device is comprised of a cylindrical filter contained

within a second cylinder. Under pressure, the sample is forced between the two

rotating cylinders, fluid is passed through the filter into the centre of the device for

removal, while the particles are forced to the outside, eliminating filter clogging (Paul et

al., 1991). In the TFF devices, the sample is forced across the filter, essentially washing the filter as it passes and prolonging the permeability of the filter. The TFF requires pre-filtration of the sample to remove suspended solids, while VFF does not. When used for concentration of adenovirus in coastal waters, the VFF method outperformed the TFF method when samples were tested by nested PCR. The VFF method resulted in three of six samples testing positive, while the TFF produced only one positive result with the same samples (Jiang et al., 2001). These methods require expensive equipment and more time than viral adsorption-elution (AE) filtration methods (Fong and Lipp, 2005).

Due to the limitations of size exclusion filtration and the cost of VFF and TFF, AE filtration is widely used. At neutral pH, viruses are predominantly negatively charged and are attracted to positively charged filters or particles (Sobsey and Jones, 1979). AMDV is negatively charged above pH 4.2 (Aasted, 1985). The AE filter technique employs a charged filter (or filter cartridge) and the manipulation of electrostatic and hydrophobic interactions. This process allows for the capture of viruses from large sample volumes (hence concentration) and final elution into much smaller volumes for detection. AE filtration is divided into two groups: those that use positively charged filters and those that use negatively charged filters with a bridging ion. Many conditions influence the adhesion of viral particles to the filter, including solution pH, solution components, viral capsid composition and filter charge.

The United States Environmental Protection Agency (USEPA, 1996) designed and recommended the 1MDS method which takes advantage of the virus's negative surface

charge at neutral pH. The sample is passed though a 1MDS positively charged glass and cellulose filter cartridge. The charge interactions result in virus particles adhering to the filter. The virus is then eluted with a proteinaceous beef extract solution of high pH (9-9.5). The alteration of pH decreases the charge attraction so that the virus is released and is eluted from the filter (Sobsey and Jones, 1979). A drawback of this method is that beef extract is suspected to contain PCR inhibitors that decrease DNA amplification by PCR (Lipp et al., 2001). Another disadvantage of the method is its low viral recovery in sea water, which results from virus particles interacting with the positive ions, and not the filter (Lukasik et al., 2000). With the addition of 0.1 M NaCl to the sample, virus adsorption decreased when measured by plaque assay (Table 1.1).

Table 1.1 Adsorption of Viruses to 1MDS filters in the Presence or Absence of 0.1 M NaCl (Adapted from Lukasik et al., 2000)

	Virus Absorption (%)			
	MS2	PRD-1	ϕX174	Poliovirus
No NaCl	96	97	29	79
0.1 M NaCl	10	13	12	7

 Although cartridge filters are recommended by the USEPA (1996), this method is used with both standard flat and cartridge filters. Recovery rates were significantly higher (p= 0.001) with flat filters were compared with cartridges for the recovery of MS2 and phi X174 bacteriophage (Polaczyk et al., 2007). Verheyen et al. (2009) also used the 1MDS method with a 1% powdered milk suspension elution for the detection of adenoviruses

and rotaviruses in 10 L samples of surface, pump and well water samples in West Africa. Of the 287 sources tested, 12.9% were positive for adenovirus and 2.1% were positive for rotavirus by qPCR. In another study, using 3% beef extract for elution, Ma et al. (1994) obtained recovery rates of 95.8 ± 12% for coxsackievirus B3 and 90.2 ± 5.9% for poliovirus from 378 L spiked tap water samples, as tested by plaque assay.

Katayama et al. (2002) designed a method for the concentration of virus particles in sea water, using negatively charged nitrocellulose HA filters. Due to the attraction of opposite charges, the positively charged divalent salt ions form a complex with the negatively charged virus and the filter, effectively linking the three. The filter is washed with H_2SO_4; the low pH (~3) alters the charge of the virus capsid so that it becomes positive. The virus itself attaches to the filter, while cations and potential inhibitors are washed away. The addition of a H_2SO_4 wash improved poliovirus recovery from below 50% to above 80% (Katayama et al., 2002). The virus elution step is carried out with NaOH, which converts the viral charge back to its original negative state, causing it to be easily eluted from the filter. This method can accommodate non sea water samples, with the addition of $MgCl_2$ to the sample, prior to filtration. In addition, this method with small variations, has been used for many viruses (Hepatitis A, astrovirus, norovirus, rotavirus, and adenoviruses) in a wide variety of water samples (tidal streams, tap, mineral, sea, river, and waste water) (De Paula et al., 2007; Victoria et al., 2009; Prado et al., 2011).

Haramoto et al. (2004) altered the method of Katayama et al. (2002) as it was not appropriate for large fresh water samples. Prior to sample filtration, 250 mM $AlCl_3$ was applied directly to the filter to mimic the salt in seawater. The Al^{3+} cations formed a complex with the virus as a result of electrostatic attractions. The acid wash and elution were carried out, as explained above.

It has been documented that the addition of salt improves viral adsorption to HA filters (Wallis and Melnick, 1967), but the mechanism involved is more elaborate that just ions simply acting as a link between the virus and the filter. The addition of salt, dependant on its ionic strength, indirectly alters the pH by hydrolyzing water. This then affects hydrophobic interactions by altering the structure of complex molecules, leading to the formation of flocs or precipitates; all of these changes can influence viral adsorption (Lukasik et al., 2000). A decrease in pH, by addition of acid, had the same increased viral adsorption affect as did the addition of $AlCl_3$ (Figure 1.1), indicating that the indirect change in pH and not the $AlCl_3$, was responsible for the improved recovery (Lukasik et al., 2000). At high pH, the addition of salt also increased hydrophobic interactions (Farrah et al., 1981).

1.7.1.1 Sample Volumes

Depending on the source of water, the concentration method used and the virus of interest, sample volumes ranged from hundreds of litres to as little as 0.5 L (Table 1.2). In general, validation or testing of filter methods tends to be conducted using small volume samples with high viral content that do not represent realistic field samples.

Field samples tend to be larger in volume with low viral concentration. Katayama et al.

(2002) used 1 L artificial seawater and viral concentrations between 300 and 770

PFU/mL to develop a concentration method. They then used this procedure to test 2 L

samples of coastal seawater for the presence of naturally occurring viruses.
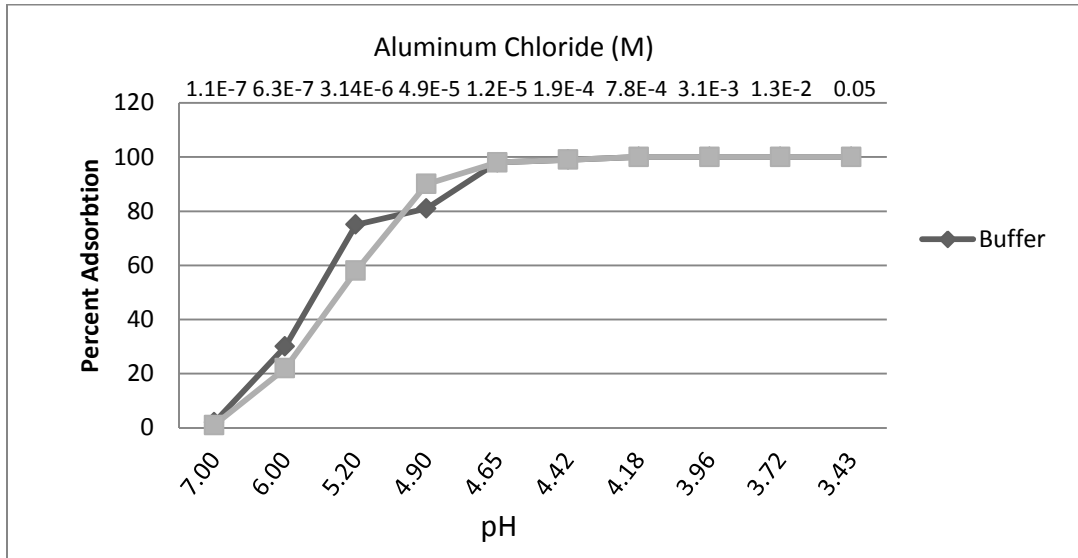
Figure 1.1 Influence of Aluminum Chloride Concentration and the Corresponding pH of a
Buffer Solution on Adsorption of Poliovirus 1 to Millipore HA filters (Adapted from
Lukasik et al., 2000)

Though no statistical analysis was preformed, Katayama et al. (2002) stated that

"increasing the volume of sea water did not affect the recovery" for this method as not

much difference was seen in poliovirus recovery rates from 1 L artificial and 50 mL

natural seawater, since rates were 50-73% and 82-95%, respectively. The similarity

between these two rates is arguable. It should be noted that these samples are difficult

to compare as the 1 L samples had lower initial poliovirus concentrations (130 to 170

PFU/mL) than the 50 mL samples (570 to 770 PFU/mL). In this experiment, recovery

rates were measured by plaque assay. Due to the size and morphology of viral plaques,

detection and counting can be difficult and may lead to assay variability. These

discrepancies in the plaque assays may have contributed to inconsistencies shown in the

Table 1.2 Summary of Sample Volumes, Water Sources, and Virus Types Concentrated
Using Electronegative Filters

Reference	Sample Volume (L)	Number of samples	Water source	Type of virus	Positive Results (%)
Katayama 2002	2	8	Sea	Norwalk G1	38
				G2	12
Haramoto 2004	10 - 532	98	Tap	Norovirus G1	4
				G2	7
Haramoto 2005	0.5	64	River	norovirus G1	53
				G2	44
				adenoviruses	45
Fuhrman 2005	1	17	Creek	enteroviruses	18
Fong 2005	0.5 - 2	30	Tidal river	BEV	37
				HEV	57
				HadV	37
Verheyen 2009	10	541	Surface and well	adenoviruses	13
				rotaviruses	2
Haramoto 2009	0.5	48	River	koi herpesvirus	4
Prado 2011	2	34	Hospital waste water	rotavirus	42.8
				adenoviruses	64.2
				norovirus 1 and 2	28.5
				hepatitis A	nd[a]

[a]not detected. All references used PCR assay for detection.

results. Haramoto et al. (2004) used small volume (40 mL MilliQ ultrapure) water

samples to test filter methods and then large volumes (100-532 L) when using the

method to concentrate and test tap water samples. Sample volume did not affect

recovery as little difference was reported between recovery yields for poliovirus in

MilliQ water from 40 mL, 500 mL, 1000 mL and 10 L, which were 99, 88, 98, and 109%,

respectively. Again, it is worth noting the variation in viral input amounts, sample volumes and the lack of statistical analysis.

1.7.1.2 Secondary Concentration

Secondary concentration is used to further reduce the sample volume from a few millilitres after AE filtration, to a smaller more concentrated volume for PCR. Methods include viral precipitation by flocculation and by centrifugal ultrafiltration (Fong and Lipp, 2005). Flocculation is conducted by the addition of flocculating chemicals that allow virus particles to clump together and precipitate out of solution. Polyethylene glycol (PEG) is an example of a commonly used flocculation agent and was used by Huang et al. (2000) to re-concentrate primate calicivirus. Another common method is ultrafiltration, which uses filters with specific pore sizes housed within centrifuge tubes. Particles smaller than the filter pores pass through while larger particles do not. Ultrafiltration, using a Centriprep concentrator (Millipore), was conducted by Katayama et al. (2002) and Haramoto et al. (2004). Haramoto et al. (2004) reported viral recovery rates between 59 and 91% for the Centriprep YM-50 filter unit.

1.7.1.3 Comparisons of Filter Methods

Comparing filter methods in the published literature is difficult as recovery is highly variable, dependent on a wide range of factors, and reported in different units (i.e. viral recovery as a %, PFU/mL and particle or copy number/L). There are a few published papers that have compared filter methods under similar conditions. Victoria et al. (2009) tested the $MgCl_2$ method (Katayama et al., 2002) in different water sources, using three

concentrations (5, 25 and 50 mM) of $MgCl_2$ for the concentration of human astrovirus and norovirus. Recovery rates varied between 0.8 ± 0.9% and 22.8 ± 17.4% for norovirus and between 0.5 (no S.D. reported) and 63.5±30.8% for astrovirus. This variability indicated that each virus required a specific concentration of $MgCl_2$and recovery was water source dependant. Haramoto et al. (2009), who developed the $AlCl_3$ method, found that the $MgCl_2$ method had better, though very low (3.6 to 7.3%), recovery of koi herpesvirus. This again indicates that viral concentration is virus specific.

Karim et al. (2009) compared the NanoCeram filter (Argonide, Sanford, FL) to 1MDS cartridges for concentration of poliovirus spiked in 100 L and 10 L samples of tap and river water. Recovery rates of poliovirus, by the NanoCeram filter and the 1MDS filter, were 51 ± 26% and 67 ± 6%, respectively in 100 L tap water and 65 ± 22% and 30 ± 11%, respectively in 10 L river water. These results indicate that recovery rate was dependant on both the type of filter and the type of water sampled. This suggests a need for filter testing with AMDV in a variety of sample types since recovery values may be unique to AMDV and the sample type.

1.7.2 Virus Detection Methods

Cell culture and polymerase chain reaction (PCR) are the two main methods that are used for the detection of viruses in environmental samples; with both having advantages and disadvantages (Fong and Lipp, 2005). Until the early 1990's, cell culture was the most common method used to determine the presence of viruses (Fong and Lipp, 2005). In this methodology, samples are inoculated into cultured cells and

incubated. Cells are examined under a microscope for signs of infection, known as

cytopathic effects (Leland and Ginocchio, 2007). The biggest advantage of cell culture

over PCR is that the former method measures viral infectivity (Richards, 1999; Fong and

Lipp, 2005). The disadvantages of cell culture are that it is very time consuming as it

can can take days or weeks for cells to grow and not all viruses will propagate in cell

culture (Fong and Lipp, 2005). Examples include calicivirus (Huang et al., 2000),

Norwalk virus (Atmar et al., 2008; Karim et al., 2009) and adenovirus (Jiang et al., 2001).

AMDV is another type of a virus that does not propagate well in cell culture (Bloom et

al., 1980), although adapted strains with low infectivity and the ability to grow in cell

culture have been developed (Bloom et al., 1994).

PCR has provided an alternative method to cell culture for viral detection. It is more

sensitive and thus allows for the detection of lower viral concentrations (Fong and Lipp,

2005). PCR has also enabled the detection of viruses that cannot grow in cell culture,

and with the use of specific primers viral detection by PCR is more precise. Primers can

be designed to target specific types of viruses or whole virus orders (Fong and Lipp,

2005). Exact viral identification, which was not always possible with cell culture, can be

made by PCR. Detection by PCR is faster and can be automated, thus decreasing the

costs of detection (reviewed in Toze, 1999). Speed, sensitivity and low cost, make PCR a

popular method for detection of viruses (Jiang et al., 2001; Haramoto et al., 2007;

Verheyen et al., 2009). One drawback of the PCR detection method is that it determines

the presence of viral nucleic acids and not the viability or infectivity of the viruses

(Leland and Ginocchio, 2007). Both protein capsid and nucleic acids must be intact and

free of damage to be infectious. For non-cultureable AMDV, PCR is the best detection

method, but results must only be interpreted as the presence of viral DNA and not

infectious virus particles.

1.8 NUCLEIC ACID EXTRACTION

The sensitivity of PCR is dependent on the quality and quantity of DNA in a sample

(Wilson, 1997). To improve PCR success, nucleic acid extraction methods are used to

increase nucleic acid concentrations and remove PCR inhibitors. Jiang et al. (2001)

reported that after viral nucleic acid extraction (GuSCN-silica beads), 3 of 6 previously

negative coastal water samples (VFF concentrate added directly to PCR) became positive

for adenovirus by nested-PCR.

1.8.1 PCR Inhibitors

Inhibitors within samples hamper amplification by impeding cell lysis, causing DNA or

primer degradation, and/or by inactivating the polymerase enzyme (Wilson, 1997). A

variety of inhibitors are present in many types of samples, including polysaccharides and

proteins in animal tissues (Perez et al., 2012), bile salts and polysaccharides in feces

(Radstrom et al., 2004), heparin, hemoglobin and hemin in blood (Wilson, 1997) and

polysaccharides in saliva (Chittick, et al., 2011). Potential components of water samples,

including organic and inorganic compounds, heavy metals, acids and humic substances,

can have inhibitory effects on PCR (Wilson, 1997). Purity is particularly important for

virus detection in environmental samples that contain low numbers of viral copies

(Griffin et al., 2003; Fong and Lipp, 2005). One of the main roles of nucleic acid

extraction is to remove any such inhibitors.

1.8.2 Conventional Extraction Methods

A variety of nucleic acid extraction procedures exist, but no single method has been

universally adopted (Lipp et al., 2001). The phenol-chloroform protocol is a traditional

nucleic acid extraction method that employs an aqueous and organic phase for the

separation of nucleic acids (Sambrook et al., 1989). Chloroform's environmental and

health risks have led to less frequent use of this method. Another widely used

technique for DNA extraction is the protocol developed by Boom et al. (1990), in which

nucleic acids bind to silica particles in the presence of guanidine thiocyanate (GuSCN), a

cell-lysing agent that inactivates nucleases. Silica beads are collected and washed with

GuSCN-containing wash buffer, ethanol and acetone. The beads are dried and nucleic

acids are eluted with aqueous low-salt buffer.

1.8.3 Commercial Nucleic Acid Extraction Kits

Commercial nucleic acid extraction kits have been designed for improved reproducibility

and have protocols that are faster and easier, compared with conventional methods.

There are many extraction kits available but no kit has been identified as being superior

for all samples or types of nucleic acids (Yang et al., 2011; Rodriguez et al., 2012).

Commercial kits are generally more expensive than the conventional methods but NA

(nucleic acid) recoveries are often higher with commercial kits (Cler et al., 2006;

Christopher-Hennings et al., 2006). Many commercial kits have been used for the

extraction of viruses from environmental samples, including Trizol (Gibco BRL), SepaGene RV-R (Sanko Jun-Yaku), RNeasy mini kit (QIAGEN), MagNa Pure Total nucleic acid extraction kit (Roche Diagnostics), and QIAamp DNA mini kit (Qiagen) (Huang et al., 2000; Katayama et al., 2002; Fuhrman et al., 2005; Haramoto et al., 2009; Verheyen et al., 2009). Differences in NA recovery rates among several commercial extraction kits have also been observed (Ribao et al., 2004; de Vries et al., 2009; Chittick et al., 2011). Although both conventional and commercial nucleic acid extraction methods have been used for the detection of AMDV (Oie et al., 1996, Farid et al., 2010; Jensen et al., 2012; Nituch et al., 2012), there is apparently no published literature on the sensitivity of any of the extraction methods when used with AMDV.

1.9 FILTER RECOVERY OF VIRUSES

Viral filtration recovery rates are highly variable and are affected by many sample characteristics, including virus type, pH and sample composition. Differences between capsid composition, and thus the virus type, affect viral adhesion to filters, resulting in different recovery rates. When tap water samples were spiked with poliovirus and calicivirus, then filtered by the 1MDS method and PEG precipitation, the rate of recovery of poliovirus was 51-55%, while calicivirus was recovered at a rate of 32-44% (Huang et al., 2000). This variation was attributed to the differences in virus types and possibly, the caliciviruses being more sensitive to high pH values. Ma et al. (1994) speculated that the difference in the virus surface charge or differences in hydrophobicity across virus type may result in different adsorption efficiency and thus, recovery.

As mentioned previously, pH is a major factor in viral adhesion to the filter and consequently, it is important to viral elution and recovery. The elution of calicivirus (Pan-1) from 1MDS filters with 3% beef extract changed from 68% to <1.0% when pH was altered from 8.5 to 10 (Huang et al., 2000). As previously mentioned in Section 1.7.1, Lukasik et al. (2000) reported that a decrease in pH improved poliovirus adsorption to HA filters, which consequently affected recovery. These results indicate the major effects of pH. The interaction effect between virus charge and pH on virus recovery remains unclear.

Sample composition and the presence of naturally occurring filter inhibitors can also impact recovery, either by blocking filter pores (Haramoto et al., 2004), by preferential binding to the virus, or by altering the affinity of virus particles to the filter (Lukasik et al., 2000; Fong and Lipp, 2005). Inhibitors can have detrimental effects on viral detection by interfering with the viral concentration step, either by reducing viral adhesion to the filter or by reducing viral elution from the filter. Inhibitors were reported as the cause of lower sensitivities not only in buffer and lab grade water, but also in tap and environmental samples. Recovery rates of Pan-1 seeded in 200 mL deionized, tap, ground and surface water samples were 94%, 73%, 67% and 64%, respectively, when measured by plaque assay after concentration with 1MDS filters and extraction with 3% beef extract (Huang et al., 2000). This variability in viral recovery between sample types is likely due to the presence of inhibitors. When Pan-1 was seeded in 0.1 M PBS, adsorption was significantly decreased and it was speculated that

the presence of Cl^- and PO_4^{3-} anions interfered with the virus binding to the filter (Huang et al., 2000).

With so many factors involved, it is understandable that recovery rates are highly variable. Fuhrman et al. (2005) indicated that recovery rate varied with virus concentration and recoveries at low viral concentrations are "fairly noisy" and not linear. At an initial spike level of 6.6 poliovirus particles/mL in both fresh and sea water, recovery was unpredictable. Mean recovery rates at concentrations $<10^4$enterovirus particles/mL were 17.3 ± 1.2% and 6.9 ± 0.4% for freshwater and sea water, respectively. Recovery rates also varied with virus type. Haramoto et al. (2009) used the $AlCl_3$ method for the recovery of polovirus and koi herpesvirus from lake water samples. Recovery rates were 56% and 3.6%, respectively. Rigotto et al. (2009) used the $MgCl_2$ method, as described by Katayama et al. (2002), for the recovery of adenovirus and hepatitis A virus in four water types. Recovery rates ranged from 10 to 100% and they concluded that recovery was dependent on both the type of virus and the water matrix.

1.10 OCCURRENCE OF FREE NUCLEIC ACIDS

The presence of free, non-encapsulated viral RNA in environmental water samples has been investigated and indicates that degradation occurs quickly. Limsawat and Ohgaki (1997) reported that RNA was degraded soon after (<30 min) seeding in raw waste water samples and Qβ coliphage RNA could not be detected by PCR after 30 min of

incubation. In another study, poliovirus RNA could not be detected in seeded samples of unfiltered seawater after 2 days of incubation at 23 °C and 4 °C (Tsai et al., 1995).

The deoxyribose sugar in DNA is more stable than the ribose sugar in RNA (Wang and Kool, 1995), therefore DNA molecules are expected to persist longer than RNA before degradation. Steps can be taken to decrease the amount of non-infectious nucleic acids present in a sample, including preparing PCR primers in regions most susceptible to degradation, pre-treating samples with enzymes to degrade free nucleic acids or damaged capsids or by using a cell monolayer prior to PCR to removed non-infectious particles (Rodriguez et al., 2012).

1.10.1 Concentration of Free Nucleic Acids by AE Filtration

There is limited literature reporting the recovery of free, non-encapsulated viral nucleic acids by the AE filter methods. It was speculated (Haramoto et al., 2004) and then confirmed (Haramoto et al., 2007), that virus particles are recovered at much higher rates than free RNA. Using the $MgCl_2$ filter method, recovery of non-encapsulated poliovirus RNA was 5.7, 12.0, 3.4 and 17.0% in MilliQ, tap, secondary-treated sewage and seawater, respectively. Recovery of polio virions using the same method and water types was 83, 79, 50 and 115%, respectively. The $AlCl_3$ method had high but variable recovery rates of RNA, 69 ± 52% in MilliQ water. These results indicated that if and when present, free RNA is concentrated at lower rates than the complete virus particle. This is also the speculated case for DNA.

1.11 LIMIT OF DETECTION AND QUANTIFICATION

When the viral concentrations of samples are low, the limit of detection (LOD) and the

limit of quantification (LOQ) become extremely important parameters. LOD calculations

are common across many fields and there are many definitions for the LOD in chemistry

and biology. Examples of LOD definitions include "the minimum single result which, with

a stated probability, can be distinguished from a suitable blank value" (McNaught and

Wilkinson, 1997) and "the lowest amount of an analyte in a sample which can be

detected but not necessarily quantified as an exact value" (Holst-Jensen and Berdal,

2004). Prichard (2001) defined LOD as:

$$LOD = (mean\ of\ blanks) + K(SD)$$

Where 'K' is the coverage factor associated with a desired confidence level and 'SD' is

standard deviation of the blank samples.

1.11.1 Limit of Detection for Standard PCR

The formula proposed by Prichard (2001) is difficult to use for standard PCR because the

results of blanks are presence/absence and therefore would not give an accurate LOD

result. For standard PCR, the LOD is the smallest amount of DNA that will produce a

visible band on the agarose gel. Samples with DNA concentrations close to the LOD may

produce faint bands or shadows, or may produce bands in intermittent replicates and

are thus difficult to accurately score. It is important to clearly define how such samples

should be handled (i.e. will faint bands be considered positive or negative?) as the main

objective of any experiment should be to treat all samples in the same manner. While

many papers have reported the detection of viral nucleic acids in clinical and

environmental samples, there are fewer publications that have referred to the presence

of faint bands (Kho et al., 2000; Thiery et al., 2001) or have discussed the LOD or

sensitivity for standard PCR (Lanciotti et al., 1992; Atmar et al., 1995). Lanciotti et al.

(1992) reported an LOD of 100 dengue virus particles based on a dilution series which

produced faint bands with 10^2 virus particles and no amplification with 10.

1.11.2 Limit of Detection for qPCR

Burns and Valdivia (2008) investigated LOD of qPCR as it relates to the detection of

genetically modified material in food products. Using computer modeling, Burns and

Valdivia (2008) evaluated the impact of cycle cut-off point (Ct 36-50) and replication

level (1-6) on the experimental and theoretical LOD of both transgenic and endogenous

soya targets in flour. Results showed that the effect of sample replication was small.

Using a cut-off value too low (Ct 36) may result in the rejection of true positive results,

and the selection of a cut-off that was too high (Ct 40) may cause false positive results.

Their work highlights the importance of defining and justifying selected cut-off points,

and indicates that cut-off points must be tailored for each assay.

Many publications report minimal or unclear information concerning the LOD used to

determine positive and negative samples. Di Pasquale et al. (2010) considered qPCR

amplification positive when Ct values were below the 50 cycles used and no

amplification was seen in the negative control (no cases of non-zero Ct values were

reported). Two negative controls were run on each plate but it was not stated if the

negative controls were water blanks or a non-template control (sample containing non viral RNA). It is also not clear if samples were replicated. When considering the work of Burns and Valdivia (2008), the cut-off value of 50 seems quite high and is presented without justification. Cler et al. (2006) used qPCR to detect GADPH DNA in paucicellular clinical samples. Samples were considered positive if the Ct value was less than that of the water blank, but it was clear how this was conducted, if mean values were used, or if editing of the blank was performed.

In experiments utilizing a standard curve, LOD is frequently reported in similar units to such standard. Volle et al. (2012) calculated LOD at 6 copies of human enterovirus (EV)/μL of sample, which was the most diluted level of the standard curve. The mean inter- assay Ct value for LOD was 40.08 (of the 45 cycles). Furthermore, Volle et al. (2012) tested 12 replicates of 2-fold serial dilutions of the virus to improve the threshold of detection. Analysis of the results with probit regression indicated that the lowest viral copy number that could be detected with at least 90% probability was 15/μL. Volle et al. (2012) did not elaborate on the discrepancy between the two LODs. A conclusion that can be drawn from the above publications is that, for qPCR, there is no sole method for setting the cut-off point and thus, LOD, and there is always a chance of false negative or false positive samples when viral concentration is low.

1.11.3 Limit of Quantification for qPCR

In some cases, the LOQ in qPCR is set at the lowest concentration of the standard curve, below that, the values are out of the linear range of qPCR and are prone to errors. The LOQ is frequently reported as viral copy number and is variable across experiments.

Volle et al. (2012) implemented a LOQ equivalent to the LOD of 15 copies of human enterovirus (EV)/μL of sample. Fuhrman et al. (2005) used a standard curve with the lowest concentration of 33 virus particles per well (25 μL reaction), but no LOQ was reported. They reported concentrations of field samples (river water) between 1.1 ± 0.37 to 23.0 ± 2.3 enterovirus particles/mL of the original sample. The standard curve was reported in particles per well and results were reported in particles/mL for the original sample prior to concentration, therefore it is difficult to decipher if an LOQ was used. Speicher and Johnson (2012) reported the LOQ as the lowest point of the standard curve at which linearity was maintained; this was equivalent to 4.85×10^3 and 3.01×10^2 copies of herpesvirus/μL in two different assays. In conclusion, as reported in the literature, LOQ is frequently equal to the lowest concentration of the standard curve, but the limit is not always clearly defined.

1.12 STATISTICAL ANALYSES

The statistical handling of the data in the comparisons of filter methods is variable. In some cases, a filter method was reported as superior if the magnitude of viral recovery or viral concentration was higher without performing any statistical comparisons (Huang et al., 2000; Katayama et al., 2002; Haramoto et al., 2004; Victoria et al., 2009; Haramoto et al., 2009).

When data are continuous, such as the result of qPCR or plaque assays, analysis of variance (ANOVA) or the t-test are the methods of choice (Wait and Sobsey, 2001; Lukasik et al., 2000; Polaczyk et al., 2007; Di Pasquale et al., 2010). For the analysis of

binary data, such as the presence or absence of a virus, or when analyzing relationships between viral presence and environmental variables, the χ^2 test (Prado et al., 2011) or binary logistic regression (Fong et al., 2005) have been used. Jiang et al. (2001) used Pearson correlation to compare the relationship between the presences of human adenovirus with coliphage concentration; however this may not have been an appropriate test because the data appeared to violate the normality assumption.

1.13 THESIS OBJECTIVES

The objectives of this thesis were to:

I.   Evaluate four different, commercially available, DNA extraction kits and identify one which was best suited for the extraction of AMDV DNA for use in downstream PCR.

II.   To develop a method for capture, concentration and detection of AMDV from spiked Millipore, tap, well and river water samples.

III. To determine the presence and concentration of AMDV in water samples collected from two Nova Scotia mink farms and surrounding water bodies.

CHAPTER 2. A COMPARISON OF FOUR DNA EXTRACTION KITS FOR THE DETECTION OF AMDV FROM SAMPLES OF MINK ORIGIN BY PCR

ABSTRACT

The success of viral DNA amplification by PCR depends on the amount and quality of DNA as well as the presence of inhibitors. Thus it is influenced by the extraction method used, particularly when viral copy number is low or samples contain inhibitory substances. Four commercial nucleic acid extraction kits were compared for quality and yield of Aleutian mink disease virus (AMDV) DNA. These kits were: Dynabeads viral extraction kit (DB) from Invitrogen, QIAamp DNA mini kit (QI) from Qiagen, MiniPrep kit (AX) from Axygen and the viral extraction kit (ZR) from Zymo Research. The quality of extracted DNA was assessed according to the success of PCR amplification and the yield which was measured by quantitative real-time PCR (qPCR). In Trial 1, DNA from spleen and fecal samples of infected mink were extracted in triplicate and 45 PCR reactions were performed with each kit. Compared with DB, DNA extracted by QI, AX and ZR was amplified by PCR in 95.6%, 53.3% and 62.2% of reactions, respectively, suggesting that DB and QI were comparable and outperformed AX and ZR. DB and QI kits were further compared by extracting DNA from identical samples in three trials. In Trial 2, AMDV-infected spleen, feces, saliva and plasma samples were assessed and viral recovery was measured by qPCR (64 reactions). In Trial 3, spleen homogenate containing 1.05×10^5 AMDV copies per µL was serially diluted by a factor of 10^1 to 10^5 and tested by standard PCR (435 reactions). In Trial 4, DNA extracted from ten of the above dilution series was tested by qPCR (146 reactions). The mean viral copy number was dependent on the initial concentration and ranged between 15 and 10.5×10^4 per µL of sample. The χ^2 goodness of fit test and logistic regression in Trial 1 and 3 showed that the DB and QI kits were not significantly different when tested by standard PCR. Analysis of qPCR results by ANOVA of qPCR results in Trial 2 indicated that the DB kit had significantly higher recovery over QI. Analysis by multiple linear regression, in Trial 4 indicated that the QI kit had significantly better performance than DB only for samples of low concentration.

2.1 **INTRODUCTION**

Worldwide, Aleutian mink disease virus (AMDV) causes the most serious malady facing

the mink industry (Hunter, 1996). The virus induces an immune complex disease that

results in significant losses to the mink industry due to increased mortality

(Alexandersen et al., 1989), decreased fertility (Hansen and Lund, 1988) and lower pelt

quality (Farid and Ferns, 2011). The virus, belonging to the family *Parvoviridae*,

possesses a 4.8 kb single stranded DNA genome contained within a non-enveloped, 18-

26 nm icosahedral capsid (Bloom et al., 1980).

The ability to detect small amounts of virus in animals and the environment is essential

for disease control. Only a small number of viral copies are needed to cause infection of

animals, therefore, the sensitivity of the detection method is especially important.

Polymerase chain reaction (PCR) has become a common method for the detection of

viruses because of its sensitivity, reproducibility, speed and low cost (reviewed in

DeBiasi and Tyler, 2004; Wang and Taubenberger, 2010). Although the sensitivity of

PCR is greater than other detection methods it can still be highly variable and is

dependent on the quantity and quality of sample DNA (Radstrom et al., 2004). Poor

quality samples tend to contain PCR inhibitors, such as polysaccharides and proteins

from animal tissues (Perez et al., 2012), bile salts and polysaccharides from feces

(Radstrom et al., 2004), heparin, hemoglobin and hemin from blood (reviewed in

Wilson, 1997) and polysaccharides from saliva (Chittick, et al., 2011). These substances

can impact PCR amplification by impeding cell lysis, causing target DNA or primer

degradation, or inactivating the polymerase enzyme (Wilson, 1997).

Commercial DNA extraction kits have been designed for faster and easier nucleic acid extraction with a higher DNA yield and quality compared with conventional methods, such as phenol-chloroform extraction. Although commercial kits are generally more costly than conventional methods, they have a higher nucleic acid recovery for genomic DNA (Cler et al., 2006) as well as viral nucleic acids (Christopher-Hennings et al., 2006) and are designed to remove or reduce PCR inhibitors.

The list of commercially available nucleic acid extraction kits is broad and no single kit has been identified as being superior for all sample sources and target nucleic acids (Yang et al., 2011; Rodriguez et al., 2012). The efficiency of nucleic acid recovery, purity of the products and thus, downstream PCR amplification success, is affected by the source of biological samples and the extraction kit used (Wilson, 1997; Ribao et al., 2004; Cler et al., 2006). Each kit is unique and the components are proprietary; different rates of nucleic acid recovery and inhibitor removal from various samples have been reported for a range of kits (Ribao et al., 2004; de Vries et al., 2009; Chittick et al., 2011).

A variety of viral DNA extraction methods have been used for detecting AMDV. The phenol-chloroform (Gottschalck et al., 1991; Oie et al., 1996) and high-salt methods (Farid et al., 2010; Farid and Ferns, 2011) have been used to extract AMDV DNA from mink tissues. In general nucleic acid recoveries are often higher when extracted with commercial kits compared to conventional methods (Cler et al., 2006; Christopher-Hennings et al., 2006). The sensitivity of these conventional methods is low for AMDV,

as shown by the inability of Oie et al. (1996) to detect AMDV by PCR in raccoon tissue homogenates, while the homogenate caused infection in mink after injection. A few commercial kits have been used recently for AMDV DNA extraction from mink tissues, including TriPure from Roche (Knuuttila et al., 2009), QIAamp Blood Mini kit from Qiagen (Jensen et al., 2011; Jensen et al., 2012) and DNeasy kit from Qiagen (Nituch et al., 2012). The relative sensitivity of these kits for AMDV has apparently not been established in the literature, nor have commercial kits been compared for their ability to extract AMDV DNA from mink-derived samples.

The objective of this experiment was to compare DNA quality, yield and sensitivity of four commercial kits that have been recommended by their manufacturers for viral nucleic acid extraction. Dynabeads Silane Viral Extraction Kit (Invitrogen, Burlington, ON) (DB), QIAamp DNA mini kit (Qiagen, Mississauga, ON) (QI), AxyPrep Body Fluid Viral DNA/RNA MiniPrep kit (Axygen Biosciences, Union City, CA) (AX) and ZR Viral DNA/RNA kit (Zymo Research, Orange, CA) (ZR) were evaluated for cost, input and output volumes, procedure duration, as well as viral recovery from various mink sources.

2.2 METHODS AND MATERIALS

2.2.1 Sample Preparation

Spleen homogenate: A 10% (w/v) homogenate was prepared from the spleens of four mink experimentally inoculated with a local strain of AMDV. Aliquots consisting of three grams of spleen tissue was mixed with 27 mL of 1X phosphate buffered saline and homogenized using a Polytron homogenizer (Kinematica AG, Switzerland) for two 30 s cycles at 15,000 rpm. The homogenates were centrifuged at 1380 x g for 10 min and

cell-free supernatants were collected. To ensure uniformity of the stock, supernatants

were pooled, thoroughly mixed, aliquoted into 1.5 mL tubes and stored at -80°C. This

homogenate was used as the source of the virus for each Trial.

Fecal matter: Fresh fecal samples were collected from 12 AMDV-infected mink by

spreading a paper towel under the animals' cages the night before collection. Each fecal

sample was transferred to a clean tube and weighed, an equal volume of RNAse-free

water was added and the mixture was vortexed to make a homogenous mixture. The

mixtures were centrifuged at 16,000 x g for 10 min, cell-free supernatants were

collected and two pools were prepared. The fecal matter sample was stored at -80 °C

until use.

Saliva: Samples were collected from eight AMDV-infected mink by rubbing sterile

calcium alginate swabs (Fisher Scientific, Ottawa, ON) inside their oral cavities. The

swab tip was cut into a 1.5 mL tube containing 600 μL of RNAse-free water. After

vortexing, the swab tips were removed prior to centrifuging at 16,000 x g for 10 min.

The cell-free supernatants from the eight mink were pooled for analysis and stored at

-80 °C until use.

Plasma: After euthanasia, a blood sample was collected into an EDTA coated tube. The

cell free plasma sample was separated by centrifugation at 1380 x g for 10 min and was

stored at -80°C until use.

2.2.2 DNA Extraction

All DNA extractions were carried out in triplicate following the protocol of the manufacturer. Briefly, DNA extraction by DB consisted of a lysis step followed by the addition of 1 μm ferromagnetic beads coated with a silica like compound. Silica beads (bound with nucleic acids) were collected using a magnetic rack. Following subsequent washing with supplied buffer, the DNA was released by heating the beads in elution buffer. The three other kits involved column separation techniques; QI employs a silica-gel membrane contained within a spin column. DNA binds to the silica membrane during washing and is then eluted in the final step. The AX kit precipitates proteins after lysis and the supernatant is applied to the AxyPrepcolumn. The ZR kit utilizes a single buffer system that lyses viruses and promotes DNA binding to the Zymo-Spin IC Column matrix. Extractions were carried out over multiple days with both the order of samples and the extraction methods randomized.

2.2.3 Viral DNA Amplification by PCR

Viral DNA amplifications by standard PCR were carried out in 15 μL reaction volumes containing 0.1% Tween 20, 1X PCR buffer, 0.2 mM dNTPs, 400 nM of each primer, 4 units of *Taq* polymerase (Invitrogen), 1.5 mM $MgCl_2$ and 1.5 μL, 2.5 μL or 3.5 μL of sample DNA. Three DNA volumes were used because viral DNA concentration could not be determined by a spectrophotometer, low sample volumes may not contain sufficient number of viruses to guarantee replication and high sample volumes may contain substances that prohibit PCR amplification. Primers were 60F: 5'-GGGTGTATGGATGAGTCCTAAA and 60R: 5'-CCCCAAGCAACGTGTACT (Farid et al., 2011).

Amplifications were carried out in a Bio-Rad thermal cycler programmed for 5 min at 95°C, followed by 35 cycles of 94°C for 30 s, 56.4°C for 30 s and 72°C for 1 min, followed by a final extension of 72°C for 6 min. Amplification success was determined by the presence of a 532 base pair band on a 1% agarose gel stained with ethidium bromide. All PCR amplifications included both a negative control (water) and a positive control (known positive sample). Negative amplification as indicated by no band was assigned a 0, while 1's were assigned to positive amplifications with visible bands. Inconclusive results from very faint bands or shadows were identified as 0.5.

2.2.4 Viral DNA Quantification by Real-Time Quantitative PCR (qPCR)

The 20 μL qPCR reaction contained 0.8 μL (400 nM) of each primer, 10 μL SYBR Green Super-mix, or Eva Green (Bio-Rad, Mississauga, ON), 2 μL sample DNA and 6.4 μL ddH$_2$0. Forward and reverse primers were 165-F: 5'- CCA ACC AAG GTA ACG CA and 165-R: 5'-CTG GAG TAT ATG GCA GTA TGT T, which amplifies a 99 bp fragment of the VP2 gene (Farid and Rupasinghe, 2012). Amplifications were performed in triplicate using a Bio-Rad iQ5 real-time PCR machine. The amplification program consisted of 3 min at 95°C followed by 40 cycles of 95°C for 15 s, primer annealing at 59°C for 10 s and extension at 72°C for 10 s. The melt-curve analysis program was run between 55°C to 95°C with 0.5°C increments, each for 30 s. qPCR amplification plots were checked to ensure every reaction had amplified to the plateau phase. Some samples showed low amplification levels or non-specific amplification. These samples judged by the amplification curves and melt curve analysis, were removed from quantification. Samples for which two of the three PCR replicates did not amplify properly were also removed. The standard

curve, which was included on every plate, consisted of 10-fold serially diluted plasmid containing the complete viral capsid protein (VP2) gene (Farid and Rupasinghe, 2012). Positive, negative and non-template controls were run on each plate in triplicate. The positive control contained previously tested AMDV positive DNA, the negative control contained mink DNA without AMDV and the non-template control contained PCR grade water. Melt curve analysis was performed after each amplification to assess PCR specificity. qPCR data was edited using a standard operating procedure (Appendix C). Samples with no amplification and those with standard deviations above 0.35 for Ct values were excluded from the analysis. Efficiency values of the standard curves were between 85% and 115% and R^2 values were above 0.95.

2.2.5 Experimental Design

Trial 1: DNA was extracted in triplicate from three dilutions of the spleen homogenate (1:0, 1:1 and 1:2 in water) and the two fecal matter samples using all four kits (DB, QI, AX and ZR). All samples were amplified by standard PCR using three DNA volumes (45 PCR reactions/kit).

Trial 2: The DNA from three spleen homogenate dilutions (1:2, 1:10 and 1:50 in water), two fecal matter, one saliva and one plasma sample were extracted by the DB and QI kits and quantified by qPCR in triplicate. Spleen and fecal matter samples were tested on four 96-well plates and saliva and plasma samples on three 96-well plates. DNA from tissues extracted by DB and QI were tested together on every plate. A total of 70 mean copy numbers (36 for DB and 34 for QI) were analyzed.

Trial 3: To test the sensitivity of DB and QI at decreasing DNA (viral) concentrations, extractions were carried out on 14 spleen homogenate samples diluted in PBS. The 14 samples were diluted by a factor ranging from 10^1 to 10^5. Both kits required the same amount of starting material, but in order to compare the two kits, elution volumes of 100 μL from the DB kit were increased to 200 μL with PCR grade water. The range of dilutions was selected to provide PCR amplifications from 100% to 0%. All extractions were amplified by standard PCR (n=435).

Trial 4: DNA that was extracted from ten of the dilution series (1/10, 1/100, 1/1000, 1/3200, 1/6400, 1/10000, 1/12800, 1/25600, 1/51200 and 1/100,000) in Trial 3 were quantified by qPCR in triplicate on five plates. Due to space limitations it was not possible to put all samples on the same plate, but to overcome plate to plate variation all samples to be compared were on the same plate. Samples of the same dilution that were extracted by DB and QI were tested on the same plate. A total of 96 replicate means were analyzed.

2.2.6 Statistical Analysis

All analyses were carried out using SAS (Statistical Analysis System) software, V9.2. PCR amplification results of the four kits were compared by the χ^2 goodness of fit test. Analysis was conducted within each source (spleen, fecal matter) because the logistic regression models that included the effects of extraction kits and source of the virus encountered only quasi-complete separation of the data points. For Trial 2, $\log_{10}$ of mean viral copy numbers were analyzed by Analysis of Variance using a model that

included the effects of extraction methods (DB, QI), source of DNA (spleen, saliva, serum, fecal matter) and their interactions. Data from Trial 3 were analyzed by the logistic regression model which included the effects of extraction methods, DNA volume and the dilution series as a continuous variable. The dilutions that provided 95% PCR success were computed for each method by Probit analysis.

Regression of $\log_{10}$ of viral copy number (LCN) on $\log_{10}$ of dilution series (LD) was computed within each extraction method in Trial 4. Normality of viral copy numbers was tested by Kolmogorove-Smirnov test and logarithm based 10 transformation was required to ensure normality. The $\log_{10}$ of dilution series linearized the regression of the LCN on LD. A multiple linear regression model included LD, the extraction methods (coded as 0 or 1) and the interaction between extraction methods and LD. The model tested for the equality of regression coefficients within each extraction method and the interaction between extraction methods and LD. To determine the equality of mean LCN at the lowest and the highest LD levels, the intercepts were set at LD-1 and LD-5, respectively.

2.3 RESULTS

2.3.1 Comparison of Four Extraction Kits

All four kits required the same input volume (200 µL), but the eluted volumes varied between 10 and 200 µL (Table 2.1). The QI kit provided the largest output volume, this may be of benefit when performing many assays but may also produce a more dilute sample. Using the DB kit was the most time-consuming (85 min per six samples) and the ZR kit was the most expensive ($5.20 per sample).

Table 2.1 Salient Features of the Four DNA Extraction Kits

Kit	Vendor	Output (μL)	Cost ($ per extraction)	Approximate duration (per six samples) min
DB	Invitrogen	100	3.57	85
QI	Qiagen	200	2.53	52
ZR	Zymo Research	10	5.20	40
AX	Axygen Bioscience	60	2.40	35

DB refers to Dynabeads Silane Viral NA, QI to QIAamp DNA Mini Kit, ZR to Zymo Research Viral DNA/RNA Kit, AX to AxyPrep Body Fluid Viral DNA/RNA MiniPrep Kit. Input volume was 200 μL for all kits. Prices are in US dollars as of March 2012.

In Trial 1 samples extracted with the DB, QI, AX and ZR were PCR amplified in 100.0%, 95.6%, 53.3% and 62.2% of the reactions, respectively when inconclusive results were considered as negatives (χ^2=42.9, P=0.0001) and in 100.0%, 97.8%, 77.8% and 80.0% of the reactions, respectively when inconclusive results were considered as positives (χ^2=18.6, P=0.0004).

Spleen samples extracted with the DB, QI, AX and ZR were PCR amplified in 100.0%, 100.0%, 62.9% and 81.5% of the reactions, respectively when inconclusive results were considered as negatives (χ^2=15.8, P=0.001) and in 100.0%, 100.0%, 74.1% and 92.6% of the reactions, respectively when inconclusive results were considered as positives (χ^2=21.3, P=0.0001). The corresponding figures for fecal matter samples were 100.0%, 88.9%, 38.9% and 33.3% when inconclusive results were considered as negatives (χ^2=12.3, P=0.006) and 100.0%, 94.4%, 83.3% and 61.1% of the reactions when inconclusive results were considered as positives (χ^2=27.6, P=0.0001). The χ^2 test

showed that the DB and QI kits were not significantly different for any of the tissues analyzed. In all analyses the AX kit was significantly different than the DB and QI kits. In 5 of 6 analyses the ZR kit was significantly different than the DB and QI kits.

2.3.2 Virus Quantification of Samples Extracted by the DB and QI Kits

In Trial 2, both the extraction method and the source of DNA had an impact on the amount of AMDV quantified (Table 2.2). The mean viral copy number per µL (taking the antilog) of extracted DNA form spleen homogenate, plasma, saliva and fecal matter by DB (340 copies/µL) was significantly higher than that for QI (157 copies/µL). There was a wide range of viral concentrations in various tissues, ranging between 15 in saliva and 2818 copies/µL in fecal matter samples (Table 2.3). The interaction between sources of the virus and extraction methods was not significant, indicating that the two kits were comparable across those sources which have a wide range of viral copies (P=0.148).

Table 2.2 Type 3 Analysis of Extraction Method and Source of DNA on Copy Number Measured by qPCR

Source	DF	Chi-Square	Pr>ChiSq
Extraction Method	1	1.7249	0.0164
Source of DNA	3	13.8121	<0.0001

Table 2.3 Least-squares Means and Standard Errors of $\log_{10}$ Viral Copy Number per µL for Source of DNA and Antilog of the Means

Source of DNA	Log Mean ± SE	Antilog
Fecal Matter	3.45±0.13[d]	2818
Plasma	1.98±0.15[b]	95
Saliva	1.18±0.15[a]	15
Spleen Homogenate	2.84±0.10[c]	692

Values bearing different superscript letters are significantly different; *P* < 0.05

2.3.3 Viral DNA Extraction Sensitivity for the DB and QI Kits as Determined by Subsequent PCR Analysis

The logistic regression analysis of the data in Trial 3 showed that dilution was significant but that there were no differences between extraction methods, DNA volume or the interaction between extraction method and dilution series (Table 2.4) after non-significant effects were removed.   Similarity between extraction methods was shown by the Probit analysis (Figure 2.1), where the 95% probability of PCR success for the DB was 1/225 (95% CI: 1/68 to 1/457) and the corresponding figure for QI was 1/267 (95% CI: 1/82 to 1/541). Odds ratios for 1.5 vs 2.5 µL of DNA was 0.72; for 1.5 vs 3.5 µL it was 0.48 and 2.5 vs 3.5 µL it was 0.66, showing a tendency (P=0.06) for higher PCR success rate with higher DNA volumes.

Table 2.4 Logistic Regression Analysis of PCR Success in Trial 3

Effects	DF	Wald Chi-square	Probability
Extraction method	1	0.04	0.84
DNA volume	2	5.75	0.06
Dilution	1	101.7	0.00
Extraction Method*Dilution	1	0.02	0.88
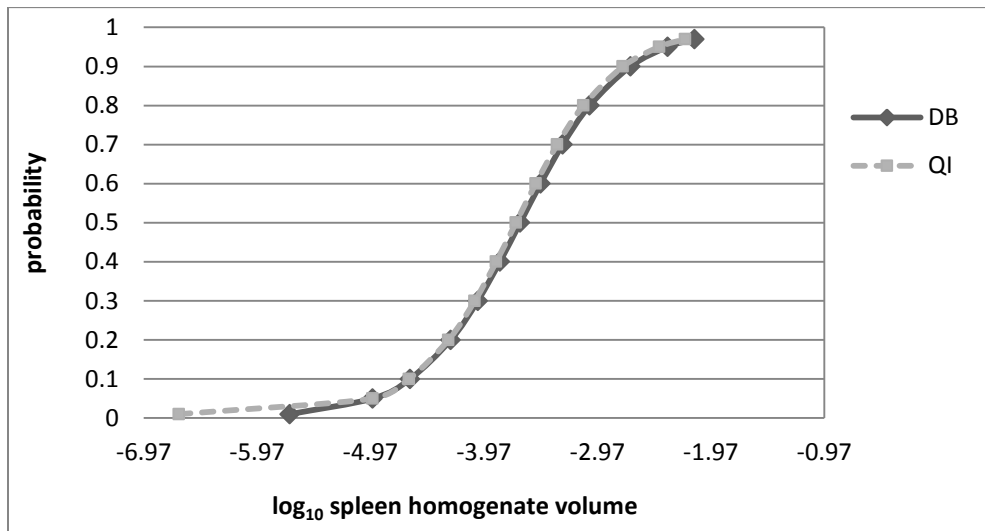
Figure 2.1 Probit Results for the Probability of PCR Amplification for DB and QI Extraction Methods versus $\log_{10}$ Spleen Homogenate Volumes

2.3.4 Virus Quantification in Diluted Spleen Homogenate Samples Extracted by the DB and QI Kits

In Trial 4, the regression equation of $\log_{10}$ of viral copy number (LCN) on $\log_{10}$ of dilution series was linear and the coefficients of variation (R^2) were high for both DB (96.1%) and QI (94.8%). Multiple regression analysis showed that the difference between the intercepts of DB and QI (0.20740, 95% CI: -0.03378 to 0.44858) was not significant (P=0.09). The intercepts at the highest dilution level were 1.29323 (95% CI: 1.18601 to 1.40044) for DB and 1.55840 (95% CI: 1.44893 to 1.66787) for QI and the difference (-0.265, 95% CI: - 0.41626 to 0.11409) was significant (P=0.001). The results imply that while the two methods were comparable at high viral concentrations, QI had a significantly higher viral recovery than the DB at the lowest viral concentration (1/100,000 dilution) (Figure 2.2). The values at the lowest viral concentration

corresponded to approximately 36.1 and 19.6 viral copy number per μL of input DNA for

QI and DB, respectively.
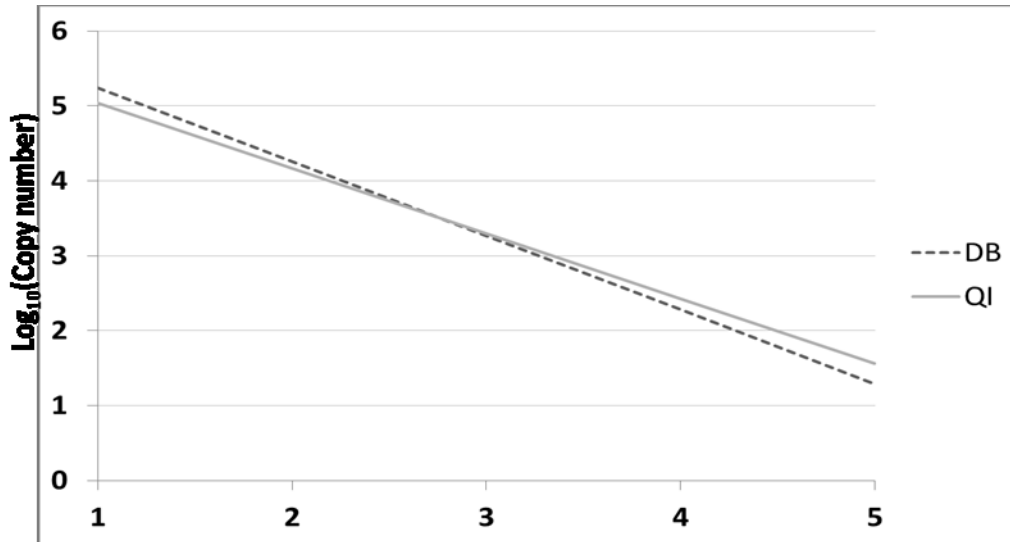
Figure 2.2. Regression of $\log_{10}$ Copy Number on $\log_{10}$ of Diluiotn of Spleen Homoginate Extracted by DB and QI Methods (Trial 4)

2.4 DISCUSSION

Spleen was selected as a sample source due to its high viral content (Bloom et al., 1985)

and frequent use as a source of the virus from mink carcasses (Knuuttila et al., 2009;

Farid et al., 2010; Farid and Ferns 2011; Jensen et al., 2011; Nituch et al., 2012; Jensen

et al., 2012). Blood serum and plasma are also common sources of AMDV DNA in live

mink (Oie et al., 1996; Manas et al., 2001). Fecal matter and saliva swabs were used

because of their ease of collection and lower stress on live mink, compared with blood

sampling by toe-nail clipping. These sources are known to contain PCR inhibitors (Perez

et al., 2012; Radstrom et al., 2004; Wilson, 1997; Chittick, et al., 2011).

The DB kit is recommended by the manufacturer for viral nucleic acid extraction from human plasma or serum, while the QI, ZR and AX kits are recommended for cell-free body fluids and tissues. However, to the best of my knowledge they have not been compared for viral recovery from feces or saliva. In this experiment, the significantly lower rates of PCR amplification success of the ZR and AX kits compared to the DB and QI were possibly due to either lower PCR inhibitor removal or lower DNA yield from spleen homogenate and feces samples.

No report was found which compared ZR or AX kits with the conventional DNA extraction methods or with any other nucleic acid extraction kits. However, the ZR kit was previously used for the detection of RNA and DNA viruses in the human respiratory tract (Fabbiani et al., 2009) and the AX kit has been used for extraction of two DNA viruses from human urine (Hu et al., 2011) as well as an RNA virus from chicken bursa tissue (Lee et al., 2011). In addition to their lower PCR success, ZR and AX had lower output (10 and 60 μL, respectively) compared with DB and QI. It should also be noted that the AX kit was the least expensive and required the least amount of time per extraction.

The DNA yield, quality and thus, the PCR sensitivity for samples extracted by the DB and QI kits were not comparable amoung all four trials. In Trial 2, higher quantities of viral DNA were extracted with DB than with QI (P=0.016), while in Trial 4 QI outperformed DB at low viral concentrations (P=0.001). de Vries et al. (2009) reported that the QIAamp Blood Mini kit outperformed DB for viral DNA (cytomegalovirus) extraction from dried

blood spot samples. Cler et al. (2006) found the QIAmp DNA Investigator kit (Qiagen) to

outperform DB for genomic DNA extraction from paucicelluar epithelial cells and

lymphocytes. The QIAamp DNA Mini kit that was used in the present work is not

identical, though similar, to those used by Cler et al. (2006) and de Vries et al. (2009).

The QIAamp DNA Mini kit does not contain carrier RNA which exists in the Investigator

kit and contains an extra buffer for tissue lysis that is not found in the Blood Mini kit.

Results from samples of low viral concentrations in Trial 4 agree with the results of de

Vires et al. (2009) and Cler et al. (2006) that the QI kit recovers more viral DNA than the

DB kit. On the other hand, results from samples of high viral concentrations in Trial 4

and results from Trial 2 do not. These divergent results between Trial 2 and 4 may be

the consequence of adjustments used to make output volumes comparable; in Trial 2 QI

qPCR results were multiplied by two and in Trial 4 DB elutions were diluted to 200 μL. In

order to compare extraction kits the output volumes must be taken into consideration,

how this is achieved may affect the sensitivity of extraction. Cler et al. (2006) used 20

μL elution volumes so that all kits they tested would have the same output volume; this

deviates from the manufacturers protocol for Dynabeads and could have resulted in

non-optimal extraction and decreased sensitivity. Differences between the results of

Cler et al. (2006), de Vries et al. (2009) and our work may also be the result of

differences between the target DNA, sample type, amplification protocols and/or input

volumes.

The DB kit was more expensive ($3.57 vs $2.53 per sample) and required more time

than the QI kit (85 vs 52 min). The output volume of DB was half that of QI (100 μL vs

200 µL). The viral concentration of the pre diluted, eluted DB sample was twice that of

the QI samples as indicated by the similarity between the two kits after adding 100 µL

water to the DB samples. The higher viral concentrations of samples extracted by DB

could provide more flexibility for downstream applications.

Detection by standard PCR of both DB and QI samples showed no amplification at the

1/51,200 dilution of the spleen homogenate. The qPCR assay however, detected the

virus at 1/100,000 dilution of the spleen homogenate as indicated by amplification

before the negative control. These results agree with literature that qPCR is more

sensitive than standard PCR (Dagher et al., 2004; Nagaraj et al., 2006). Thus, although

more expensive, qPCR is recommended when samples contain low quantities of target

DNA.

In this experiment three volumes of DNA were used for standard PCR because it was not

possible to accurately measure viral DNA concentration with a spectrophotometer.

Furthermore, samples with low viral concentrations may not contain sufficient number

of viruses to guarantee replication, while high sample volumes may impair PCR

amplification because of high amounts of inhibitors. However, the tendency for higher

amplification success with higher DNA volumes in Trial 3 suggests that both DB and QI

efficiently removed inhibitory materials.

The use of three DNA volumes allowed for the investigation of the impact of faint,

indeterminate, difficult to call, shadow bands that are commonly observed at low viral

loads. The indeterminate data were analyzed as both positive and negative. For the AX

and ZR kits, PCR amplification success ranged widely between positive and negative inclusion analysis (77.8% vs 53.3% and 62.2% vs 80.0%) for AX and ZR, respectively. The DB and QI kits had a smaller range (100.0% vs 100.0% and 97.8%vs 95.6%) indicating that there were more faint bands for the AX and ZR kits and they may have been operating close to their limit of inhibitor removal.

In conclusion, the nucleic acid extraction kit used can affect the sensitivity of viral detection. When comparing kits, the method used to obtain similar input and output volumes is important. When output volume was equated computational (i.e. multiplied by 2) DB was superior over QI, but when output volume was equated by dilution, QI was superior over DB at very low viral concentrations and only when qPCR was used. DB and QI were superior to the AX and ZR kits. Both PCR success and DNA concentrations were independent of the source of DNA, i.e. spleen homogenate, saliva, plasma and fecal matter. Based on cost, procedure duration and sensitivity at low concentrations the QI may be a suitable replacement for the DB kit currently used in our laboratory.

CHAPTER 3. COMPARISON OF THREE FILTER METHODS FOR THE CONCENTRATION OF ALEUTIAN MINK DISEASE VIRUS IN WATER SAMPLES FOR SUBSEQUENT PCR ANALYSIS

ABSTRACT

In order to measure viral particles at low concentration in the environment, samples will require concentration prior to analysis. In this experiment, three filter methods, positive charged 1MDS filter with beef extract, negatively charged HA filter with $MgCl_2$ or $AlCl_3$, were evaluated for their efficiency to concentrate AMDV from 500 mL spiked samples of Millipore, tap, well and artificial river water. Filter methods were compared in three trials. In Trial 1, nine decreasing volumes of virus stock (1000 to 0.001 µL) were added to only Millipore water. After concentration by the three methods and DNA extraction using Dynabeads, the presence of AMDV was detected by standard PCR and the results were analyzed using the logistic regression model. In Trial 2, four volumes (10, 5, 1 and 0.1 µL) of virus stock were spiked into all four water types and the presence of AMDV was detected by standard PCR. Results were analyzed using the logistic regression model. In Trial 3, 500 mL samples of Millipore, well and tap water were spiked with 10 µL and 0.1 µL of virus stock, filtered by the 1MDS and $MgCl_2$ methods, quantified by qPCR and analyzed by ANOVA, using three different qPCR cut-off values. Results from Trial 1 indicated that when tested with Millipore water, the 1MDS filter method produced the highest rate of PCR amplification success, followed by the $MgCl_2$ and the $AlCl_3$ methods. In Trial 2, the 1MDS and $MgCl_2$ methods were comparable for PCR success in Millipore, tap and well water samples and were superior over the $AlCl_3$ method which was discontinued. Millipore water samples had higher amplification success than tap and well water samples and viral detection form river water was very poor. The decreased efficiency of the filter methods with tap, well and river water indicates the presence of inhibitors of PCR or filtration. In Trial 3, AMDV recovery was dependent on the filter method, the water type and the initial amount of virus added. The interaction between water source and virus stock volume and the interaction between filter method and virus stock volume were also significant at the various cut-off values. The 1MDS method had higher viral recoveries and was significantly different from the $MgCl_2$ method, when analyzed for two of the three cut-off values. Recovery rates were highly variable and ranged between 403% and 1.8% for Millipore and well water samples, respectively. This work indicates that the 1MDS filter method is somewhat better than the $MgCl_2$ method and superior to the $AlCl_3$ method for the capture of AMDV, though recovery rates depend on sample type.

3.1 INTRODUCTION

The detection of viruses in water samples is both necessary and complex. Many viruses are transmitted by the ingestion of contaminated water and infect the host, via the fecal-oral route (Fong and Lipp, 2005). In order to protect against diseases, it is important that water be free of virus particles prior to use by plants, animals or humans. The detection of virus particles in water samples is challenging due to low virus concentrations. Without host replication, as in the environment, viral concentration is significantly affected by dilution.

Low viral concentrations in water samples require concentration for successful detection. Several techniques have been developed for both viral concentration and viral detection. Adsorption-elution (AE) filtration for viral concentration and PCR for viral detection are two of the commonly used methods (Fong and Lipp, 2005). The AE filter method utilizes electrostatic attraction to concentrate viruses (USEPA, 1996; Katayama et al., 2002; Haramoto et al., 2004). At a neutral pH, negatively charged virus particles bind to positively charged filter membranes (Sobsey and Jones, 1979), after which, viruses are eluted into smaller volumes when the attraction is weakened, or reversed, via the manipulation of pH (Lukasik et al., 2000). Electropositive filters are required by the USEPA (1996) method for viral recovery and are commonly used (Abbaszadegan et al., 1993; Borchardt et al., 2004; Verheyen et al., 2009). With the addition of divalent ions, negatively charged filters have also been employed. The ions

act as a bridge, or link, between the viruses and the filter. Negative filters tend to have higher virus recovery in sea and turbid water (Katayama et al., 2002). They have been used to concentrate many viruses, such as animal rotaviruses from tap water (Gratacap-Cavallier et al., 2000), human enteroviruses from freshwater creeks (Fuhrman et al., 2005), bovine enteroviruses and human adeonviruses from river samples (Fong et al., 2005) as well as hepatitis A from tidal rivers (De Paula et al., 2007). Negative filters have been used with $MgCl_2$ added to the sample (Katayama et al., 2002) and $AlCl_3$ added directly to the filter (Haramoto et al., 2004).

Although viral detection by PCR cannot distinguish between infectious and non-infectious viruses, it has the advantage of increased sensitivity, specificity and more rapid result turn-around than cell culture (Fong and Lipp, 2005). PCR is particularly useful for the detection of viruses, such as AMDV, that do not propagate well in cell culture (Bloom et al., 1980).

Viral recovery and thus, the sensitivity of detection, is influenced by many factors other than the method used for concentration and detection. Sample characteristics, such as pH, the presence of inhibitory substances and the type of virus, are all significant factors. Under different pH conditions, virus particles can be either negatively or positively charged, depending on the isoelectric points of their proteins (Gerba et al., 2008). Therefore, a change in solution pH and its effect on viral surface charge influences both viral adhesion and elution from filter membranes (Huang et al., 2000; Lukasik et al., 2000). Also, inhibitory substances within a sample can decrease viral

recovery by hindering filtration or PCR. PCR inhibitors, such as humic acids, interfere

with amplification by impeding cell and viral lysis, causing nucleic acid degradation, or

decreasing the function of the polymerase enzyme (Wilson, 1997). Filter inhibitors, such

as Cl^- and PO_4^{3-} ions, interfere with virus-filter binding, as was reported when calicivirus

was seeded in 0.1 M PBS (Huang et al., 2000). DNA extraction and secondary

concentration are two steps taken to mitigate amplification inhibitors (Radstrom et al.,

2004). With so many factors involved, viral recovery rates vary widely, from as low as

0.8 ± 0.9% for norovirus in sea water (Victoria et al., 2009), to as high as 94 ± 13% for

Pan-1 in deionized water (Huang et al., 2000).

Defining detection limits is a crucial step in the identification of viruses in environmental

samples by qPCR. Poorly defined limits can lead to false negative or false positive

results (Burns and Valdivia, 2008). Conventional limits of detection calculations, which

use the values of blanks or negative controls, cannot be used for qPCR because blanks

and negative controls may have variability, or high non-zero values (Burns and Valdivia,

2008). Though seldom discussed in the literature, defining the cut-off point at which

amplification can be distinguished or quantified from background is essential for

accurate results. Methods used to define the limit of detection (LOD) include the use of

determined Ct value cut-offs (Di Pasquale et al., 2010), or any sample that amplifies

before the blank, (i.e. the sample had a Ct value less than that of the blank) (Cler et al.,

2006). Some publications do not identify the procedures used for defining LOD

(Fuhrman et al., 2005). The limits of quantification (LOQ) have been set by

implementing the LOD (Volle et al., 2012) or by determining the lowest point in the

standard curve for which the curve remains linear (Speicher et al., 2012).

The purpose of this experiment was to test three filter methods, for their ability to

capture of AMDV from 500 mL spiked water samples and to identify a method for use

when testing environmental water samples. A positive 1MDS flat filter using the USEPA

(1996) protocol, a negative HA filter using the Katayama et al. (2002) protocol and the

same HA filter using the Haramoto et al. (2004) protocol were tested for their suitability

to concentrate the virus from Millipore, tap, well and artificial river water. Viral

detection was conducted by standard and qPCR; viral concentrations were quantified by

qPCR.

3.2 MATERIALS AND METHODS

3.2.1 Sources of Water

Four types of water were used in this experiment, including Millipore, tap, well and

artificial river (referred to as river for the remainder of this chapter) water. The

Millipore water was obtained from a Milli-Q Millipore filtration system (Millipore, USA).

Un-chlorinated tap water was taken from a laboratory tap in the bio-security laboratory

of the Cox Institute. To ensure freshness, tap water samples were collected after the

tap had run for five minutes. Two 10 L aliquots of well water were collected from a local

well in Bible Hill, NS and stored in polyethylene containers at 4 °C until use. River water

was made in the laboratory and contained dissolved organic carbon and chemicals

similar to natural river water (Morel and Hering 1993; Sangi et al., 2002; Taylor, 2009).

An organic stock was prepared by mixing 6 L of Millipore water and 270.19 g of peat

moss (Pro-gro, Annapolis Valley Peat Moss Co., Ltd, NS) for 1.5 h using a magnetic stir

bar and stir plate. The separation of solids from liquid was conducted with Whatman

541 filter paper, housed in a 10 cm filter holder, under vacuum. After filtration, no

settling was observed. One litre of the organic stock was mixed with 14 L Millipore

water and the chemicals listed in Table 3.1. The final solution had a pH of 6.8 and was

stored at 4 °C until required. Mineral water analysis of the Millipore, tap, well, organic

stock and the river water was conducted by Quality Evaluation Division of the Nova

Scotia Department of Agriculture.

Table 3.1 Chemicals Used for Artificial River Water

Chemical	Amount in milligrams/15 L	Molar Mass	mmol
$CaCl_2 \cdot 2H_2O$	109.6	147.0154	0.75
$NaHCO_3$	315.4	84.00679	3.75
$MgSO_4 \cdot 7H_2O$	185.4	246.4755	0.75
$KHCO_3$	32.7	100.11533	0.33

3.2.2 Spiking of Water Samples With Virus Stock

Previously prepared AMDV positive spleen homogenate (Chapter 2.2.1) was used as a

virus stock to spike water samples. All spleen homogenate was stored at -80 °C prior to

use. All inoculated water samples were incubated at room temperature for 30 to 60

min prior to filtration. The amount of the virus stock varied among trials and is further

discussed in Section 3.2.6.

3.2.3 Primary Virus Filtration

Primary filtration was conducted using the 1MDS, the $MgCl_2$ and the $AlCl_3$ methods outlined below. All filtrations were carried out in a laminar flow fume hood, using a 47 mm glass vacuum filter holder (Fisher Scientific) attached to a 500 mL vacuum flask. Gentle vacuum was used to provide flow rates of approximately 100 mL/min and the elutant was filtered into a clean 25 mL flask. Between samples, all glassware was rinsed with a disinfectant (Virkon, Aquameirk, St-Nicolas, QC) to eliminate possible cross contamination of subsequent samples, cleaned with soap and water and rinsed with dH_2O. Waste water was mixed with bleach and held at room temperature for 24 hrs, or autoclaved, prior to disposal.

3.2.3.1 1MDS Filtration Method

The 1MDS method was based on the protocol outlined by the United States Environmental Protection Agency (USEPA, 1996) with the exception that a flat filter instead of a cartridge was used. The 500 mL sample was passed through a 47 mm 1MDS flat filter (Kinecor, Quebec). Both the sample beaker and funnel walls were rinsed with Millipore water to ensure that all viral particles were collected. The bottom flask was replaced with a 25 mL flask, containing 50 µL of 100x Tris-EDTA (TE) buffer. The virus was eluted with 3 mL beef extract (Sigma-Aldrich, Oakville, ON) (1.5% w/v, 0.05 M glycine pH 9.5), followed by an additional 2 mL of beef extract. This was applied directly to the filter and then placed under vacuum conditions.

3.2.3.2 MgCl$_2$ Filtration Method

The MgCl$_2$ method was described by Katayama et al. (2002). Briefly, 5 mL of 2.5 M MgCl$_2$ was added to the 500 mL sample and filtered through a HA filter (0.45 µm pore size, 47 mm diameter, Millipore). After the sample beaker and funnel walls were washed using Millipore water, the filter was rinsed with 200 mL 0.5 mM H$_2$SO$_4$ (pH 3.0). The bottom flask was replaced with a 25 mL flask, containing 50 µL 100x TE buffer and 25 µL 100 mM H$_2$SO$_4$ (pH 1.0). Virus elution was conducted, with a slight variation from the Katayama et al. (2002) protocol. Instead of one 5 mL application of 1.0 mM NaOH (pH 10.8), 3mL followed by 2 mL was used.

3.2.3.3 AlCl$_3$ Filtration Method

Two mL of 250 mM AlCl$_3$ was passed through a HA filter prior to the water sample, as per Haramoto et al. (2004). The same washing and elution procedures were carried out, as per the MgCl$_2$ method above.

3.2.4 Secondary Concentration

The concentrated eluted samples (~5 mL) were transferred to an Amicon Ultra-4 filter (100,000 NMWL, Fisher Scientific) for further concentration and spun at 1380 x g (Model 228, Fisher Scientific) until the sample volume was between 150 and 200 µL. Final volumes were adjusted to 200 µL with Millipore water and stored at 4 °C until DNA extraction using Dynabeads. To ensure minimal DNA degradation, extractions were conducted on the same day as filtration.

3.2.5 DNA Extraction and PCR Amplification

DNA extraction was conducted, using Dynabeads, as reported in Chapter 2. Standard

PCR using a Bio-Rad thermal cycler and evaluation of amplification success were

conducted as reported in Chapter 2. Briefly, three DNA volumes were used for

amplification and success was determined by the presence of a 532 base pair band on a

1% agarose gel. No band indicating negative amplification was given a 0 and 1's were

assigned to positive amplifications. In addition to the evaluation outlined in Chapter 2,

when a sample produced two negative and one inconclusive result across the three

volumes it was re-amplified. qPCR was conducted as per Chapter 2 on a Bio-Rad iQ5

real-time PCR machine.

3.2.6 Experimental Design

3.2.6.1 Trial 1 - Comparison of Three Filter Methods using Spiked Millipore Water Samples and Detection by Standard PCR

To compare the viral recovery of the three filter methods, one of nine volumes (1000,

100, 10, 5, 1, 0.1. 0.01, 0.005, and 0.001 µL) of virus stock was added to 500 mL

Millipore samples and filtered by the 1MDS and $MgCl_2$ methods. Due to poor

amplification, only the six largest virus stock volumes were filtered by the $AlCl_3$ method.

All filtrations were carried out in triplicate, unless otherwise indicated in Appendix A.

3.2.6.2 Trial 2 - Comparison of Three Filter Methods using Spiked Millipore, Tap, Well and River Water Samples and Detection by Standard PCR

The 1MDS, $MgCl_2$ and $AlCl_3$ filter methods were further compared, using tap, well and

river water spiked with four volumes (10, 5, 1 and 0.1 µL) of virus stock in 500 mL

samples. Preliminary results of the $AlCl_3$ method resulted in unacceptably low recovery of virus (Appendix B) therefore this method was removed from the trial mid-way through the experiment. With some exceptions, the 1MDS and $MgCl_2$ methods were conducted with each water type, at each dilution, in triplicate (Appendix B).

3.2.6.3 Trial 3 - Quantification of Viral Copies Concentrated by the 1MDS and $MgCl_2$ Filter Methods in Spiked Millipore, River, Tap and Well Water Samples

To compare viral recovery rates of the filter methods, Millipore, river, tap and well water samples were spiked with 10 and 0.1 µL of virus stock and filtered using the 1MDS and $MgCl_2$ methods, in triplicate. Each sample was amplified in two different reactions (different plates) and all samples of the same filter replicate were amplified on the same plate. Only two virus stock volumes were used due to space limitations of the number of samples that could be analyzed on each plate. The qPCR results were manually edited, as explained in Appendix C. The mean viral copy number of the two or three PCR replicates for each sample, on each plate, was used for statistical analysis. One plate was retested due to high standard deviation of the last dilution on the standard curve (above 0.35). At least 18 amplifications (3 filter replicates x 3 PCR replicates x 2 plates) were performed for each sample.

3.2.7 Statistical Analyses

3.2.7.1 Trial 1 - Comparison of Three Filter Methods Using Millipore Water by Standard PCR

A logistic regression model in SAS was carried out to investigate the association of virus stock volumes, filter method and DNA volume, with the probability of PCR success. The

model related the probability of positive PCR results to the filter method, virus stock volume and DNA volume. Analysis was conducted with confirmed faint-bands grouped with positive results and with a total of 270 observations.

3.2.7.2 Trial 2 - Comparison of Two Filter Methods Using Millipore, Tap, Well and River Water by Standard PCR

Logistic regression models, within water types, were used to evaluate the effects of filter method, DNA volume and the $\log_{10}$ of virus stock volumes on standard PCR success. The model including all parameters did not converge, therefore analyses were conducted within each water type and the interactions were removed. A total of 72, 96, 132, and 162 observations were used for the analysis of Millipore, river, tap and well water respectively. Analysis of river water was not reported because the large amount of negative results produced a quasi-complete separation of data points.

3.2.7.3 Trial 3 - Quantification of Viral Copies Concentrated by the 1MDS and MgCl$_2$ Filter Methods in Millipore, River, Tap and Well Water

The data were analyzed by the PROC GLM of SAS using the following model; $Y_{ijkl}=\mu+ S_i+ F_j+ V_k+ SF_{ij}+ SV_{ik}+ FV_{jk}+e_{ijkl}$, where μ is the overall mean, S_i is the effect of the ith source of water, F_j is the effect of the jth filter method, V_k is the effect of the kth virus stock volume, SF_{ij}, SV_{ik} and FV_{jk} are the two-way interactions and e_{ijkl} is the random error. Only 8 of the 24 river samples amplified. As in Trial 2, river water samples were removed from the final analysis. Three different cut-off values were used to evaluate viral recovery of each filter method from each water source. In Analysis 1 (all data), all samples that amplified with Ct values below than of the negative controls were used (79

observations). In Analysis 2, results fewer than 10 copies per μL were removed (62 observations). Ten was selected as a cut-off value because it was midway between the last dilution used to produce the standard curve and the next value which was not included on the standard curve. In Analysis 3, a more stringent quantification was used by setting the cut-off value of 22 viral copies, this was the lowest value used to produce the standard curve (53 observations). Normal distribution of the data was achieved by taking the log_{10} value of the mean of copy number. In cases where the viral copy number was zero, 1 was assigned to accommodate log_{10} transformation. Interactions were not included in the model in Analysis 3 because some subclasses had no observations and means could not be estimated. Recovery rates were calculated using the unadjusted mean viral copy number of the virus stock, experimental samples at the three cut-off values and an adjustment factor. A virus stock volume of 200 μL was used for DNA extraction, therefore it contained 20 or 2000 times more viral copies than the input volume used for filtration (10 or 0.1 μL).

$$\text{Recovery rate} = 100 \times \frac{\underline{\text{Mean copy number of sample/μL)* (20 or 2000)}}}{\text{Mean copy number in virus stock/ μL}}$$

3.3 RESULTS

3.3.1 Water Analysis

Mineral water analysis of the Millipore, tap, well, organic stock (used to make the river water) and the river water was conducted by Quality Evaluation Division of the Nova Scotia Department of Agriculture (Table 3.2). Tap water had the highest concentrations for all components tested, except for zinc, which was highest in well water. The pH was lowest for the organic stock at 4.2 and pH of the other samples fell between 5.66 and

7.83. Conductance for well, river, organic stock and Millipore water was 36.6%, 8.6%,

5.7% and 1.6% respectively, expressed as a percentage of tap water. Alkalinity and

conductance had a 20 and 60 fold difference across the test samples and were lowest

for the organic stock and Millipore water, respectively. Total hardness ranged from 0.04

mg/L in Millipore to 244.52 mg/L in tap water. The tap water sample was the only

sample to have a Nitrate + Nitrite-N value above the detection limit (1.00 mg/L) and was

3.10 mg/L. Mineral concentrations were lowest for Millipore water, followed by river,

well and tap. Dissolved organic carbon (DOC) was measured using an Auto Analyzer

(Table 3.3) and ranged from 28.30 mg/L to 0.94 mg/L in organic stock and river water,

respectively.

Table 3.2 Analysis of Water Used to Test Filter Methods

	Millipore	Tap	Well	Organic stock	River
pH	5.66	7.68	7.83	4.2	7.01
Nitrate + Nitrite -N (mg/L)	<=1.00[*]	3.10	<=1.00[*]	<=1.00[*]	<=1.00[*]
Conductance (mmhos)	10	614	225	35	53
Alkalinity (mg/L)	15	110	79	<=5.5[*]	11
Chloride (mg/L)	<=10[*]	101	17	<=10[*]	<=10[*]
Total Hardness (mg/L)	0.04	244.52	104.49	0.94	9.18
Calcium mg/L)	<=0.1[*]	80.47	38.86	0.12	1.51
Copper (mg/L)	<=0.01[*]	0.02	0.02	<=0.01[*]	<=0.01[*]
Iron (mg/L)	<=0.01[*]	0.02	<=0.01[*]	0.02	<=0.01[*]
Magnesium (mg/L)	<=0.1[*]	10.58	1.81	0.16	1.31[*]
Manganese (mg/L)	<=0.01[*]	<=0.01	<=0.01[*]	<=0.01[*]	<=0.01
Sodium (mg/L)	<=0.1[*]	26.59	4.52	1.43	5.82
Sulfate (mg/L)	<=0.1[*]	37.30	10.67	1.94	5.23
Zinc (mg/L)	<=0.01[*]	0.02	0.18	<=0.01[*]	<=0.01[*]
Potassium (mg/L)	<=1.00[*]	1.47	<=1.00[*]	<=1.00[*]	<=1.00[*]

[*]below detection limit

Table 3.3 Dissolved Organic Carbon in Tap, Well, Organic Stock and River Water Samples used to Test $AlCl_3$, $MgCl_2$ and 1MDS Filter Methods

	Tap	River	Well	Organic Stock
DOC Concentration (mg/L)	1.10	0.94	1.30	28.30

3.3.2 Trial 1 - Comparison of Three Filter Methods using Millipore Water by Standard PCR

The initial logistic model included virus stock volume, filter method, DNA volume, the interaction between virus stock volume and filter method, and the interaction between filter method and DNA volume. The interactions were not significant and were removed from the final model. The effects of filter method and virus stock volume on PCR success were highly significant, but the effect of DNA volume was not (Table 3.4). The raw data summary is reported in Appendix A.

Table 3.4 Type 3 Analysis of Filter Method, Virus Stock Volume, and DNA Volume with Millipore Water by Standard PCR

Effect	DF	Chi-Square	Pr>ChiSq
Filter method	2	31.3478	<.0001
Virus stock volume	1	31.0599	<.0001
DNA volume	2	1.4418	0.558
Virus stock volume * Filter method	2	3.4177	0.1811
Filter method * DNA Volume	4	1.8591	0.7616

The odds ratio estimates indicated that, when adjusted for other terms in the model, the $MgCl_2$ method had approximately an 8-fold higher probability of PCR success (1/0.12) than $AlCl_3$ (Table 3.5). The 1MDS method had 1.8-fold higher probability of PCR

success than the $MgCl_2$. Consequently, the odds of PCR success for $AlCl_3$ was 15-fold

lower than the 1MDS. These fold increases indicated that, when testing with Millipore

water, the 1MDS filter method resulted in the highest rate of PCR amplification success,

followed by the $MgCl_2$ and the $AlCl_3$ methods.

Table 3.5 The Odds Ratio Estimates with 95% Confidence Interval for the Three Filter
Methods Using Millipore Water

Effect	OR Point estimate	95% CI
Filter method Al vs Mg	0.121	0.047-0.311
Filter method 1MDS vs Mg	1.861	0.893-3.879
Filter method Al vs 1MDS	0.065	0.025-0.170
DNA vol 1.5 vs 2.5	0.728	0.332-1.593
DNA vol 1.5 vs 3.5	0.625	0.286-1.363
DNA vol 2.5 vs 3.5	0.858	0.399-1.848

For each filter method, the probit procedure estimated the probability of PCR

amplification success at different viral stock dilutions (Figure 3.1). Over the entire range

of viral stock concentrations, the $AlCl_3$ method required higher amounts of virus stock

than both the 1MDS and $MgCl_2$ methods to produce the same probability of

amplification success. The confidence intervals for the 1MDS and $MgCl_2$ methods

overlapped for the entire range. To obtain a 95% probability of PCR success 6.6, 8.4 and

84.7 µL of virus stock volume was required for the $MgCl_2$, 1MDS and $AlCl_3$ methods. For

a 50% probability, 0.1, 0.02 and 7.5 µL of virus stock volume was required for the $MgCl_2$,

1MDS and $AlCl_3$ methods, respectively. Cross over of the 1MDS and $MgCl_2$ methods

occurred between 4.14 and 5.03 µL of virus stock (Figure 3.1).
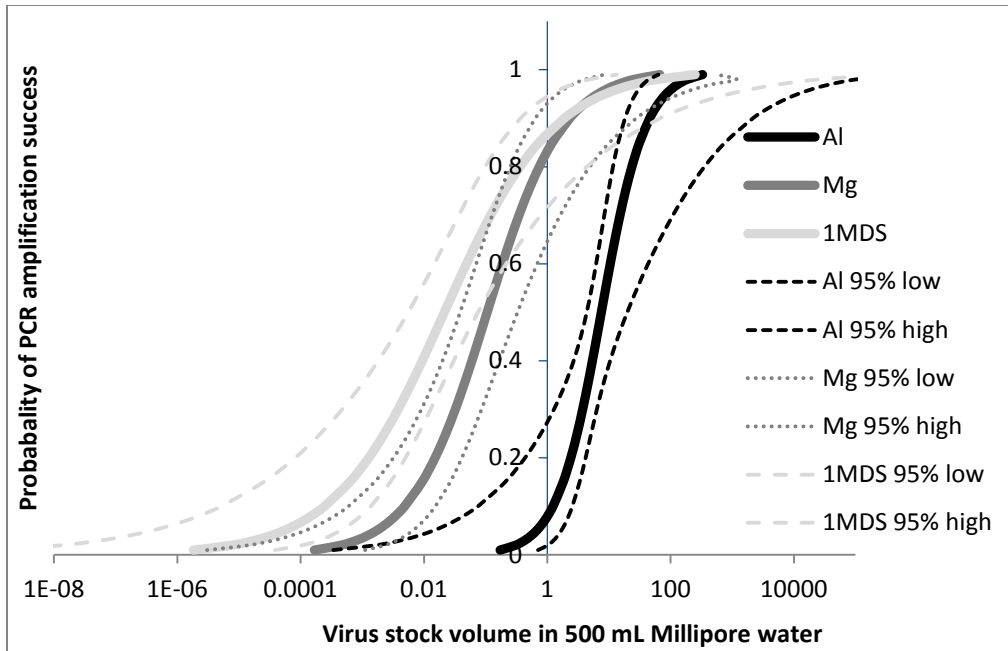
Figure 3.1 Probit Results for the Probability of PCR Amplification versus Virus Stock Volumes for the 1MDS, $AlCl_3$ and $MgCl_2$ Filter Methods with Millipore Water. Solid lines indicate the means and dotted lines represent the 95% Confidence Interval.

3.3.3 Trial 2 - Comparison of Three Filter Methods using Millipore, Tap, Well and River Water by Standard PCR

Poor amplification success of the river water samples produced many negative results in the data set resulting in a quasi-complete separation of the data points when performing logistic regression analysis. This condition brought into question the results of the statistical model employed. Therefore, river water samples were excluded from the analysis of filter methods. As previously mentioned (Section 3.2.7.2), the $AlCl_3$ filter method was discontinued midway through this experiment. Filter method and DNA volume did not influence PCR success in Millipore, tap and well water samples (Table 3.6, 3.7, 3.8). The virus stock volume impacted results for tap and well water samples, but not Millipore water, because, for many Millipore samples virus recovery was successful. Raw data indicated that Millipore water samples had much higher

amplification success than tap and well water samples (Appendix A, B). These results

may indicate that PCR inhibitors were present in tap and well water or, that the filter

methods were less efficient with these sample types.

Table 3.6 Type 3 Analysis of Filter Method, DNA Volume and Log_{10} of Virus Stock Volume
on PCR Success for Millipore, Tap and Well Water Samples

Effect	DF	Millipore		Tap		Well	
		Chi-Square	Pr>ChiSq	Chi-Square	Pr>ChiSq	Chi-Square	Pr>ChiSq
Filter method	1	0.1126	0.7372	2.2194	0.1363	0.3464	0.5562
DNA volume	2	2.6386	0.2673	5.9932	0.0500	4.0710	0.1306
Log of virus stock volume	1	0.6884	0.4067	7.0568	0.0079	24.3355	<.0001

The odds ratio estimates further indicated that samples filtered by the $MgCl_2$ method

had the same probability of amplification success, compared to the 1MDS method for

Millipore (Table 3.7), tap (Table 3.8), and well (Table 3.9) water, respectively. For tap

water in Trial 2, DNA volume was approaching significance (p=0.05) (Table 3.6). Samples

with 3.5 µL DNA had a 2-fold increase in the probability of PCR success, versus samples

with 2.5 µL DNA (Table 3.8). This trend was also observed in Chapter 2. As would be

expected, the amount of virus stock volume affected PCR success in tap and well water

samples. This was not evident in Millipore samples because at the levels tested, the

majority of samples were positive.

Table 3.7 Odds Ratio Estimates with 95% Confidence Interval for the 1MDS and MgCl$_2$ Filter Methods Using Millipore Water

Effect	OR Point estimate	95% CI
Filter method 1MDS vs Mg	0.797	0.213-2.991
DNA vol 1.5 vs 2.5	0.269	0.048-1.511
DNA vol 1.5 vs 3.5	0.424	0.091-1.962
DNA vol 2.5 vs 3.5	1.577	0.237-10.488
Log of virus stock volume	1.423	0.619-3.273

Table 3.8 Odds Ratio Estimates with 95% Confidence Interval for the 1MDS and MgCl$_2$ Filter Methods Using Tap Water

Effect	OR Point estimate	95% CI
Filter method 1MDS vs Mg	0.569	0.271-1.195
DNA vol 1.5 vs 2.5	0.659	0.268-1.619
DNA vol 1.5 vs 3.5	0.326	0.131-0.809
DNA vol 2.5 vs 3.5	0.494	0.203-1.203
Log of virus stock volume	2.132	1.220-3.728

Table 3.9 Odds Ratio Estimates with 95% Confidence Interval for the 1MDS and MgCl$_2$ Filter Methods Using Well Water

Effect	OR Point estimate	95% CI
Filter method 1MDS vs Mg	1.236	0.611-2.499
DNA vol 1.5 vs 2.5	0.424	0.178-1.013
DNA vol 1.5 vs 3.5	0.510	0.214-1.216
DNA vol 2.5 vs 3.5	1.203	0.517-2.799
Log of virus stock volume	4.755	2.559-8.835

3.3.4 Trial 3 - Quantification of Viral Copies Concentrated by the 1MDS and MgCl$_2$ Filter Methods in Millipore, Tap and Well Water

Filter method, water type and virus stock volume had varying effects on viral recovery, across the different cut-off values (Table 3.10). The interaction between the sources of

water and filter methods was not significant for any of the analyses. The interaction

between source of water and virus stock volume was significant for all data (Analysis 1),

the filter method by virus stock volume was significant with a cut-off value of 10

(Analysis 2). The significant interactions caused inaccuracies in the means of the main

effects, yet these means are presented to allow for the deduction of some general

conclusions from the data. There was insufficient data for an investigation of

interactions in Analysis 3.

Table 3.10 The F Values and Levels of Significance for Sources of Variation in Each
Analysis

		Analysis 1 All data above blank		Analysis 2 Cut-off of 10[¶]		Analysis 3 Cut-off of 22[¶]	
Source of variation	DF	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Filter method	1	6.00	0.017	0.07	0.794	6.46	0.014
Source of water	2	10.22	0.000	3.07	0.055	7.41	0.002
Virus stock volume	1	117.66	0.000	15.57	0.000	19.22	0.000
Source* Filter	2	2.26	0.112	1.47	0.240	-	-
Source * Virus stock Volume	2	5.95	0.004	1.21	0.306	-	-
Filter * Virus stock Volume	1	1.22	0.273	8.23	0.006	-	-

[¶]The $\log_{10}$ of results was used for analysis, - Indicates that implementing the cut-off
value resulting in removal of all data.

The 1MDS method had a higher viral recovery, compared with the $MgCl_2$, differences

were significant in Analyses 1 and 3, but not significant for Analysis 2 (Table 3.11). The

mean of $\log_{10}$ viral copy number, by the 1MDS method in Analysis 1, was 1.81

(corresponding to 65 viral copy number/µL), compared with 1.47 (30 viral copy

number/µL) for the $MgCl_2$ method. This indicates that the 1MDS method recovered 2.2

fold more viruses than the $MgCl_2$, when all data were analyzed (Table 3.11). The mean

of $\log_{10}$ viral copy number by the 1MDS method, in Analysis 3, was 2.26 (182 viral copy

number/µL), compared with 1.94 (87 viral copy number/µL) for the $MgCl_2$ method.

Again indicating that the 1MDS method recovered 2.1 fold more viruses than the $MgCl_2$,

when the most stringent method of analysis was used (Table 3.11).

Table 3.11 Least-squares Means and Standard Errors of $\log_{10}$ Viral Copy Number per µL
for the Filter Method, Water Source and Virus Stock Volume in Each Analysis and Antilog
of the Means

	Analysis 1 All data above blank	Analysis 1 Antilog	Analysis 2 Cut-off of 10[¶]	Analysis 2 Antilog	Analysis 3 Cut-off of 22[¶]	Analysis 3 Antilog
Filter method						
1MDS	1.81 ± 0.09^a	65	2.06 ± 0.09^a	115	2.26 ± 0.09^a	182
$MgCl_2$	1.47 ± 0.10^b	30	2.01 ± 0.14^a	102	1.94 ± 0.10^b	87
Water source						
Millipore	2.06 ± 0.11^a	115	2.22 ± 0.09^a	166	2.37 ± 0.10^a	234
Tap	1.47 ± 0.12^b	30	1.83 ± 0.12^a	68	1.80 ± 0.12^b	63
Well	1.38 ± 0.12^b	24	2.05 ± 0.20^a	112	2.12 ± 0.14^{ab}	132
Virus stock volume						
0.1µL	0.89 ± 0.10^a	8	1.69 ± 0.16^a	49	1.76 ± 0.14^a	58
10µL	2.38 ± 0.09^b	240	2.38 ± 0.07^b	240	2.43 ± 0.07^b	269

[¶]The $\log_{10}$ of viral copy number per µL was used for analysis. Values in the same cell
bearing different superscript letters are significantly different; $P < 0.05$.

The source of water was significant in Analyses 1 and 3 and approached significance in

Analysis 2 (p=0.055) indicating that quantification was significantly affected by the

samples matrix. In Analysis 1, the mean viral copy number for well and tap water

samples were comparable (24 and 30 viral copy number, respectively) and were 21%

and 26% of that of the Millipore water samples (115 viral copy number) (P<0.05). The

corresponding values were 63% and 41% in Analysis 2, which were not statistically

different. In Analysis 3, the mean viral copy number for Millipore water (234/µL) was significantly greater than that of the tap water (63/µL), but the mean viral copy number of the well water (132/µL) was not different from the other two. Mean viral copy number for tap and well water samples were 56% and 27%, respectively, of that of the Millipore water. These results indicated the presence of materials, in well and tap water samples, that may interfere with viral recovery, or act as PCR inhibitors.

 As expected, across all three analyses, the virus stock volume was significant for mean viral copies and samples seeded with 10 µL virus stock had more viral copies than those with 0.1µL (Table 3.11). Although the input viral copy number in 10 µL stock samples was 100 times higher than that in 0.10 µL stock samples, this ratio was 30.8, 4.9 and 4.4 in Analyses 1, 2 and 3, respectively. This could suggest a limit of virus absorption to the filters or a lower DNA recovery, using Dynabeads, at higher DNA concentrations.

The means of the source of water by virus stock volume subclass in Analysis 1 showed that the means of viral recovery from 10 µL virus stock volume samples were significantly greater than those from 0.10 µL virus stock volume samples, for the three water sources (Table 3.12). However, the lowest mean viral copy number from the 0.10 µL virus stock volume was obtained from the well water, while the lowest viral recovery from 10 µL virus stock volume was observed from the tap water. This resulted in significant interaction between the water sources and virus stock volumes. The ratios of viral recovery from 10 µL virus stock spiked samples over 0.1 µL virus stock spiked

samples theoretically should be 100 but were 14.8, 13.8 and 144.2 for Millipore, tap and

well water samples, respectively.

Table 3.12 Least-squares Means and Standard Errors of $\log_{10}$ Viral Copy Number per µL
for the Water Source by Virus Stock Volume Subclass for Analysis 1 and Antilog of the
Means

Source of water	0.1 µL virus stock	0.1 µL virus stock Antilog	10 µL virus stock	10 µL virus stock Antilog
Millipore	1.48 ± 0.16^c	30	2.65 ± 0.16^e	447
Tap	0.91 ± 0.19^b	8	2.04 ± 0.16^d	110
Well	0.31 ± 0.17^a	2	2.46 ± 0.16^{de}	288

Values bearing different superscript letters are significantly different; $P < 0.05$.

The means of filter method by virus stock volume subclass in Analysis 2 indicated that

when filtered by the 1MDS method, means of viral recovery from 10 µL virus stock

volume samples were significantly greater than those from 0.10 µL virus stock volume

samples (Table 3.13). When filtered by the $MgCl_2$ method, the means between 0.1 and

10 µL virus stock volumes were not significantly different. At the smaller virus stock

volume, means were not significant between the filter types, while at the higher volume

the 1MDS method was significantly higher. The ratios of viral recovery from 10 µL virus

stock spiked samples, over the 0.1 µL virus stock spiked samples were 14.9, 14.1 and 1.7

for 1MDS and $MgCl_2$ filter samples, respectively.

Table 3.13 Least-squares Means and Standard Errors of $\log_{10}$ Viral Copy Number per µL
for the Filter Method by Virus Stock Volume Subclasses for Analysis 2 and Antilog of the
Means

Filter	0.1 µL virus stock	0.1 µL virus stock Antilog	10 µL virus stock	10 µL virus stock Antilog
1MDS	1.48 ± 0.14^a	30	2.63 ± 0.10^c	427
$MgCl_2$	1.89 ± 0.26^{ab}	78	2.14 ± 0.10^b	138

Values bearing different superscript letters are significantly different; $P < 0.05$.

The viral recovery was calculated using the virus stock mean of 57110 ± 15964 viral copy number/μL, which was obtained from 20 mean estimates on seven PCR plates (Tables 3.14, 3.15 and 3.16 for Analyses 1, 2 and 3, respectively). Recovery rate was numerically greater from 0.1 μL virus stock than 10 μL virus stock, from all water sources and both filter methods in all three analyses, which may indicate a limited capacity of filter methods for viral capturing. Viral recovery was the highest for Millipore water and the lowest for tap water for 10 μL virus stock volume, in both filter methods in all three analyses. For the 0.1 μL virus stock volume, the highest viral recovery was obtained by the 1MDS method from tap water (143.6%) and by the $MgCl_2$ method, from Millipore water (402.7%) in Analysis 1. A similar pattern was observed in Analyses 2 and 3, where recovery rates could be estimated. While viral recovery rates from samples spiked with 10 μL virus stock ranged between 2.8% ($MgCl_2$ method, tap water, Analyses 1 and 2) and 48.8% ($MgCl_2$ method, Millipore water, Analysis 3), the estimates often exceeded 100% in cases where samples were spiked with 0.1 μL virus stock volume. These results could imply that the DNA recovery kit has limitations when testing samples that contain high amounts of viral DNA.

Table 3.14 Unadjusted Means of Viral Copy Number and Recovery Rates of the Filter Methods, Analysis 1

	1MDS				$MgCl_2$			
Source	10 μL	Recovery %	0.1 μL	Recovery %	10 μL	Recovery %	0.1 μL	Recovery %
Millipore	1227	43.0	30	105.0	1197	41.9	115	402.7
Tap	238	8.3	41	143.6	79	2.8	4	14.0
Well	439	15.4	6	21.0	289	10.1	0.5	1.8

Viral copy number per μL for virus stock was 57,110

Table 3.15 Unadjusted Means of Viral Copy Number and Recovery Rates of the Filter Methods, Analysis 2

Source	1MDS				MgCl$_2$			
	10 µL	Recovery %	0.1 µL	Recovery %	10 µL	Recovery %	0.1 µL	Recovery %
Millipore	1227	43.0	43	150.6	1197	41.9	132	462.2
Tap	238	8.3	48	168.1	79	2.8	15	52.5
Well	439	15.4	19	66.5	289	10.1	-	-

- Indicates that implementing the cut-off value resulting in removal of all data and therefore no recovery could be calculated

Table 3.16 Unadjusted Means of Viral Copy Number and Recovery Rates of the Filter Methods, Analysis 3

Source	1MDS				MgCl$_2$			
	10 µL	Recovery %	0.1 µL	Recovery %	10 µL	Recovery %	0.1 µL	Recovery %
Millipore	1227	43.0	59	206.6	1394	48.8	132	462.2
Tap	238	8.3	62	217.1	90.0	3.15	-	-
Well	439	15.4	-	-	289		-	-

- Indicates that implementing the cut-off value resulting in removal of all data and therefore no recovery could be calculated

3.4 DISCUSSION

3.4.1 Water Source

The components in, and characteristics of, water samples can hinder viral filtration by blocking the filter (Haramoto et al., 2004) or by altering the virus particles' affinity for the filter (Lukasik et al., 2000). These components can also inhibit PCR by altering cell lysis, degrading DNA or affecting the amplification enzyme (Wilson, 1997). To evaluate the filter methods over a variety of water types, several water sources were used. Millipore water was used as the control because it was assumed to be free of any

substance that could interfere with virus recovery or PCR amplification. Tap and well water were selected to represent possible water types found on mink ranches. The river water was made in the laboratory in an attempt to allow comparison between the results and known chemical composition. Ideally, natural river water would have been used but, because composition of natural water sources vary largely from location to location and over time, samples taken from one river would not have represent all rivers. Azoulay et al. (2001) reported different mineral concentrations across the same city, while Brylinsky (2011) found mineral concentration variation, over time, in the same lake. Without extensive sampling and comprehensive analysis, such variations would make it difficult to correlate viral recovery results to water composition.

As expected, Millipore water had below detectable amounts of minerals. Hardness, alkalinity and conductance, which are related to dissolved minerals, were also very low. Amongst the four water sources studied, tap water contained the highest concentrations of calcium, chloride, iron, magnesium, sodium, sulfate and potassium. Tap water also had the highest conductance, hardness and alkalinity. Nitrate and Nitrite-N are naturally occurring ions produced from the oxidation of nitrogen by micro-organisms (Health Canada, 2012), were highest for tap water but were still below the guidelines for Canadian drinking water quality of 10 mg/L (Health Canada, 2012). The source of Campus tap water was not municipally supplied, but from a local reservoir which is fed from a well. This water underwent no treatment (Phil Talbot, personal communication). All parameters measured for Bible Hill well water fell below that of Campus tap water, except zinc. Differences between mineral composition of the water

obtained from the well in Bible Hill and the Campus water are not unexpected, as well

water is unique to its specific location and the surrounding mineral compositions.

Azoulay et al. (2001) reported variations in mineral concentrations of tap water

between different sources within the same city. In San Jose, California, calcium levels

varied from 9 to 60 mg/L, among three waters sources. In Columbus, Ohio, sodium

levels were 10 and 51 mg/L at two sources. A 1983 study conducted in New Brunswick

reported nitrate levels in municipal water ranging from <4.4 mg/L to as high as 44 mg/L.

A similar study conducted in Nova Scotia, with 143 municipal water samples, reported a

range of 0.05 mg/L to 2.0 mg/L for nitrate (Health Canada, 1987). The nitrate plus

nitrite-N results of water used in this experiment were below the 1.00 mg/L limit of

detection for Millipore, well and river and 3.10 mg/L for tap water. The tap water value

is within the range of nitrate levels reported in NB and just above that of the range of

NS, although the previous studies only reported nitrate, and not nitrate plus nitrite-N

rates.

It was initially thought that this experiment would be able to relate the success of virus

detection with water characteristics of the samples. With only 4 measures for each

characteristic (4 water types) and the variability in PCR success this was not possible.

In this experiment, mineral concentrations of the river water were below that of the

well and tap water samples. Calcium, magnesium, sodium, sulfate, hardness and

conductance were higher in river water than Millipore water, though the alkalinity in tap

water was lower than that in the Millipore water. The pH, alkalinity, conductivity and

hardness of river water were within the range reported by Taylor (2010) from ten lakes in the Carlton River watershed (the same area where the majority of NS mink ranches are located). Both hardness and conductivity were very close to the means of these parameters reported by Taylor (2010) (Table 3.17). The concentrations of calcium, magnesium, sodium and sulfate minerals in river water were again within the range reported for lakes sampled in Digby and Yarmouth counties (Taylor, 2010). Copper, iron, manganese, zinc and potassium were not added to river water and were therefore, below the concentrations reported by Taylor (2010). In conclusion, if the minerals had been added, the river water used in this experiment is within the range of values reported for the Carlton River watershed.

Table 3.17 Comparison of River Water to Carlton River Watershed samples (Taylor, 2010)

	River	Carlton River watershed samples (Taylor, 2010)	
		Range	Mean
pH	7.01	4.30-7.50	6.23
Nitrate + Nitrite -N (mg/L)	<=1.00	<0.01-3.40	0.18
conductance (mmhos)	53	34.70-303.00	54.09
Alkalinity (mg/L)	11	<1.0-67.4	3.84
Chloride (mg/L)	<=10	5.70-36.00	9.64
Total Hardness (mg/L)	9.18	4.60-49.80	8.50
Calcium mg/L)	1.51	0.90-11.70	1.82
Copper (mg/L)	<=0.01	<2-23.00	0.40
Iron (mg/L)	<=0.01	<50-1943.00	574.76
Magnesium (mg/L)	1.31	0.50-5.00	0.99
Manganese (mg/L)	<=0.01	8.00-2770.00	142.55
Sodium (mg/L)	5.82	3.40-20.80	5.74
Sulfate (mg/L)	5.23	<5-11.00	0.16

Zinc (mg/L)	<=0.01	<5-11	1.42
Potassium (mg/L	<=1.00	<0.5-6.8	0.58

The DOC content of water measures dissolved organic compounds, including humic

substances (Thurman, 1985), that have been reported as PCR inhibitors (Wilson, 1997).

The DOC concentrations of the three water sources were comparable (0.94 mg/L for

river water to 1.30 mg/L for well water). The concentrations were within the range for

natural waters, which ranged from 0.7 mg/L in ground water to 33 mg/L in bog water

(Thurman, 1985), but results were slightly lower than typical river water and more

characteristic of a pristine stream. Although results were within the expected range,

confidence in the DOC results was low. River water was made, using 1 L of organic stock

diluted to 15 L and therefore, the DOC of river water should be approximately $1/15^{th}$ of

the DOC of the stock, equivalent to approximately 1.87 mg/L. For reasons unknown the

observed value was only half of the expected value (0.936 mg/L).

3.4.2 Effects of Filter Methods on Virus Recovery

Millipore water samples, filtered by the 1MDS method, had the highest rate of PCR

success, followed by the $MgCl_2$ and $AlCl_3$ methods (Table 3.5). The lack of interaction

between filter methods and viral stock dilution showed that the differences among filter

methods were consistent over the entire range of viral concentrations tested (from

1000 μL to 0.001 μL in 500 mL sample). The 1MDS method also outperformed the

$MgCl_2$ when viral recovery was quantified using a cutoff above the blank and a cutoff of

22 viral copies (Trial 3, Analysis 1 and 3, Table 3.10). But, the two methods were not

different within the three water types in Trial 2, or in Trial 3 with cutoff 10. It can be

concluded that the 1MDS filter generally outperformed the $MgCl_2$ method.

This work is apparently the first comparison of filter methods for the detection of AMDV

in water samples and thus, there is no literature with which to directly compare the

results. The finding that the 1MDS filter method is better than the $MgCl_2$ and $AlCl_3$

methods (in some trials) is contradictory to the results of Katayama et al. (2002) and

Haramoto et al. (2004), who, upon developing the $MgCl_2$ and $AlCl_3$ methods (using

poliovirus in laboratory grade water and sea water), found them to outperform the

1MDS method. These results cannot be directly compared to this experiment as

recovery is dependent on many factors, including virus type, sample pH and sample

composition. Sample pH affects the viruses' affinity for the filter (Lukasik et al., 2000)

and different water compositions may contain filtration and PCR inhibitors (Fong and

Lipp, 2005). Virus type is the factor most likely responsible for the difference between

the results of Katayama et al. (2002) and Haramoto et al. (2004) and those found in this

experiment. Viruses have unique surface charges that affect the interaction between

the virus and the filter (Ma et al., 1994; Huang et al., 2000). The differences in capsid

composition and isoelectric points between poliovirus (an RNA virus) and AMDV

particles (Murray and Parks, 1980; Aasted, 1985) would lead to different recovery rates.

There were two procedural variations between this experiment and that of the

literature (Katayama et al., 2002; Haramoto et al., 2004). In this experiment a smaller

volume secondary filtration apparatus, Amicon Ultra-4 was used versus the Centriprep

YM-50 (Millipore). Also, in this experiment the Dynabeads kit was used for nucleic acid

extraction while SepaGene RV-R (Sanko Jun-yaku) and QIAamp viral RNA mini kit (QIAGEN) were used by Katayama et al. (2002) and Haramoto et al. (2004). These differences may have also lead to differences in recovery.

Haramoto et al. (2007) reported that the $AlCl_3$ method had high recovery of free, non-encapsulated nucleic acids. It is possible that the virus stock in this experiment contained only minimal amounts of free DNA, which resulted in low PCR success. The results of the probit analysis from Trial 1 and the raw data in Trial 2 (data were not analyzed), support the speculation that the $AlCl_3$ method is less efficient than the $MgCl_2$ method for water samples containing small quantities of viruses (Haramoto et al., 2004). Another factor that might have influenced the results could be the relatively small number of tests that were performed for a situation with a large degree of variation. Viral capturing, viral concentration, DNA extraction and PCR amplification are a series of processes that are inherently subject to large variations; inconsistent results from different experiments, conducted under various situations, are not expected.

The cutoff point in qPCR also has a large effect on the conclusions reached in any experiment and inappropriate cutoff values can result in false negative or false positive results (Burns and Valdivia, 2008). Different cutoff values have been used by Cler et al. (2006), Di Pasquale et al. (2010), Volle et al. (2012) and Speicher et al. (2012). The absence of a universally accepted procedure lead to quantification in Trial 3 using three cutoff points (all data above blank, 10 viral copies/µL, and 22 viral copies/µL). Increasing the cutoff values from all data above the blank, to 10 and 22, resulted in

more samples with low viral copy numbers to be considered zero. This affected the main effects and interactions (Table 3.10). Filter method, source of water and virus stock volume were significant in Analyses 1 and 3. The interaction of filter method by virus stock volume was significant in Analysis 2.

Viral recoveries by the 1MDS and $MgCl_2$ filter methods were comparable for the three water sources, as shown by a lack of interaction (Trial 3, Table 3.10). The analysis of interactions between filter methods and sources of water has not been included in the statistical models in the literature; although both factors have been compared separately (Lukasik et al., 2000; Katayama et al., 2002; Haramoto et al., 2004; Huang et al., 2000). The interaction of filter methods by virus stock volume was significant, using a cutoff of 10 viral copies (Trial 3, Analysis 2, Table 3.10), where $MgCl_2$ tended to outperform 1MDS at low viral concentrations, but the reverse was true for higher viral concentrations (Table 3.14). The difference between Analysis 2 (cutoff 10) and Analysis 1 and (all values above the blank) was the conversion of low viral recoveries to zeros. This adjustment magnified the difference between viral recoveries of the two methods at low viral concentration. There is no information in the literature with which to compare these results. Since no interaction was observed between filter method and viral stock concentration in the case of Millipore water (Table 3.4), it may be concluded that the type of water plays a role in viral recovery at low viral concentrations.

3.4.3 Effects of Filter Methods on Virus Recovery Rate

Recovery rates for the 1MDS and $MgCl_2$ methods varied from 8.3% to 217.1% and from 1.8% to 462.2%, respectively (Table 3.14, 3.15 and 3.16). Recoveries over 100% are further discussed in Section 3.4.5. Obviously, virus recovery rates are variable for different viruses and experiments. The variability observed in this experiment is much higher than that reported by Haramoto et al. (2004) who described adsorption rates (recovery rates would be equal to or lower than adsorption) for the poliovirus, with 1MDS and $MgCl_2$ methods, between 33% and 98% and 66% and 75%, respectively. Huang et al. (2000) reported recovery rates of 51% to 55% and 32% to 44% for poliovirus and calicivirus in tap water samples, when concentrated by the 1MDS method. Using the 1MDS method, Victoria et al. (2009) reported recovery rates of human norovirus and astrovirus of 22.8±17.4% and 63.5±30.8% in mineral water. With the $AlCl_3$ method, Haramoto et al. (2009) reported recovery rates of 7.3% for Koi herpesvirus from laboratory grade water samples. The differences in recovery rates can, in part, be attributed to virus type, water source and the initial virus concentrations. The large standard deviation presented both in the literature and in this experiment is most likely due to the cumulative effect of errors over the many stages of the filtration and detection process.

3.4.4 Effects of Water Source on Virus Recovery Rate

Although viral recovery from Millipore, tap, river and well water sources was measured in Trial 2, the analysis was conducted within water types because analysis across water types caused quasi-complete separation of the data points. Therefore, recovery rates

between water types could not be compared. As a result of many amplification failures

with the river water samples, there was a quasi-complete separation of the data points

which produced an unreliable analysis and river water samples were removed from the

trial. In Analysis 1 of Trial 3, recoveries from tap and well water were significantly lower

than those from Millipore water, with means of $\log_{10}$ virus copy numbers/μL of 1.47,

1.38, and 2.06, respectively (Table 3.11). In Analysis 3 of Trial 3, recovery for Millipore

water was significantly higher than for tap water, with means of $\log_{10}$ of virus copy

numbers/μL of 2.37 and 1.80, respectively. The differences between virus copy

numbers/μL across water types may be attributed to their different components and

their interference with viral recovery or PCR amplification. This result was similar to the

work of Huang et al. (2000), who reported improved viral recovery of the Pan-1 virus

from deionized water, compared with tap or ground water. Natural water samples are

known to contain PCR inhibitors, such as organic compounds, including humic acids and

divalent ions (Wilson, 1997; Griffin et al., 2003).

The presences of inhibitors were speculated to have caused the low amplification

success observed in river water samples (Trial 2). All parameters measured for the river

water were within the range of the other samples tested; analyses of mineral

composition and DOC did not identify any features that could be linked to PCR failure. It

is speculated that the river water contained a non-measured component that interfered

with viral filtration or PCR amplification. The results of this experiment indicated that

neither the 1MDS filter method, the $MgCl_2$ filter method, the secondary concentration

method, nor the Dynabeads extraction were capable of removing all inhibitors that were present in the samples.

3.4.5 Effects of Virus Amount on Recovery Rates

Even for samples of the same water type, there was high variability between recovery rates when different amounts of virus stock were added (Tables 3.14, 3.15, 3.16). The recovery rates for samples with 0.1 and 10 µL virus stock volumes, filtered by the 1MDS method, were 43.0% and 206.6% in Millipore water and were 8.3% and 217.1% in tap water, respectively. Fuhrman et al. (2005) reported that recovery rate of poliovirus varied with concentration; in fresh water, at low concentrations (6.6 viral copies/mL), recovery was unpredictable and not linear, while at concentrations $<10^4$ enterovirus particles/mL, recovery was 17.3%. This published finding is similar to the results of this experiment; at low concentrations (0.1 µL virus stock), means had higher standard errors, 0.89 ± 0.10, 1.69 ± 0.16, and 1.76 ± 0.14 $\log_{10}$ virus copies/ µL, compared with the higher concentrations (10 µL virus stock), 2.38 ± 0.09, 2.38 ± 0.07 and 2.43 ± 0.07 $\log_{10}$ virus copies/ µL, for Analyses 1, 2 and 3, respectively (Table 3.11).

As mentioned in Section 3.3.4, the ratios of viral recovery were not proportional to the amount of virus stock added; samples with 10 µL virus stock did not have 100 times more virus than the samples with 0.1 µL virus stock (Table 3.12). There are two possible explanations for this. Firstly, although not reported, filters may have a limited capacity or upper limit for viral absorption so that after some concentration, viruses do not bind with the filter and pass through. This explanation is less likely as similar filters have

been used for much larger volumes (Table 1.1); Haramoto et al. (2009) recovered

326,000 KHV particles, Katayama et al. (2002) recovered 350,000 poliovirus particles,

using 1MDS filters. If limited capacity is in fact the issue, a filter with a larger diameter

could be implemented; thus, an increased surface area would allow more sample to

pass though the filter before the limit of viral absorption was reached.

The more probable explanation is that the Dynabeads extraction kit may have a limit of

DNA extraction so that samples with low concentrations have higher recovery rates than

samples with high concentrations. This speculation is supported by an Invitrogen

publication (Kleveland et al., 2008) which reported higher viral recovery for samples of

low viral concentrations. To lessen this problem, recovery should be calculated using a

virus stock sample that was diluted prior to extraction, rather than the concentrated

virus stock sample that was used in this work. This dilution prior to extraction

procedure was not followed because, at the time of the experiment, the problem was

not recognized. By comparing experimental amounts of virus recovered to the virus

stock of lower concentrations, the limitations of the extraction method may be

decreased or alleviated and thus, result in more accurate recovery rates. It should also

be noted that the recovery rates reported in this document are the combined effects of

both the filter method and secondary concentration. Haramoto et al. (2004) reported

high variability in the recovery of poliovirus, by secondary concentration (Centriprep

YM-50, Millipore), ranging from 59-91%, over four samples. To better understand

where AMDV viral loss is occurring, future work should evaluate the recovery rate of the

Amicon Ultra-4 filter.

3.4.6 Conclusion

Recovery rates of AMDV were variable for spiked water samples. The 1MDS filter method was superior or comparable with the $MgCl_2$ filter method. The $AlCl_3$ method was the least successful. As indicated in the literature, water impurities reduced recovery rate and Millipore water samples had the highest recovery, compared to well, tap and river. Further testing is recommended to optimize the method and improve viral recovery rates. Future work should strive to identify what components lead to lower recovery and at what point in the process virus particles are lost.

CHAPTER 4. DETECTION OF AMDV IN NOVA SCOTIA WATER SAMPLES

ABSTRACT

Aleutian mink disease virus (AMDV) causes the most significant malady facing the mink industry today. This persistent virus infection results in major financial losses for mink ranchers in Nova Scotia and worldwide. Even after thorough disinfection, ranch re-infection is common. Both wild animals and contaminated environments are speculated to be sources of re-infection. In this experiment, 16 one litre samples were collected from various locations of the Carlton River watershed and 11 water samples were collected from two infected mink ranches in western Nova Scotia, all were tested for AMDV. The procedure employed included virus concentration by 1MDS filtration, secondary concentration, DNA extraction by Dynabeads, detection by standard and real-time PCR and quantification by real-time qPCR. The virus was not detected in any of the watershed samples. Absence or low number of viruses in natural water samples and the presence of PCR inhibitors are the suspected causes of non-detectable AMDV. Four of the six samples (one waterline and three runoff) from ranch 1 and the pond lagoon sample from ranch 2 were positive for AMDV. The three runoff samples from ranch 1 were quantified and contained 3.35×10^4, 2.22×10^4 and 1.68×10^4 virus copies/mL. Viral copy numbers in the other two positive samples were below the level of quantification. The detection of AMDV in these samples identified possible sources for ranch animal re-infection and virus transmission to wildlife via ranch runoff. The identification of samples that contain AMDV will allow for improved virus management including the obstruction of viral re-entry onto ranches.

4.1 INTRODUCTION

The mink industry is of significant economic importance to NS; in terms of dollars, mink pelts are currently the most valuable agricultural export (Devanney and Reinhardt, 2011). The number of pelts produced, as well as pelt values, has steadily increased over the past several years (Statistics Canada, 2011). Many of the fur farms in NS, concentrated in Digby and Yarmouth counties, have been infected with AMDV (Newman

and Reed, 2006; Farid et al., 2012). The majority of NS ranches use a test and kill

strategy to control the virus. This practice has only reduced the prevalence of infection

and has not eradicated the virus (Farid et al., 2012).

The original source of the AMDV to ranches is unknown, but exposure to contaminated

environmental components, such as soil and water, are amongst the speculated causes

of virus persistence (Farid et al., 2012). Recent discussions with some NS mink ranchers

indicate that they believe ranch re-infection occurs after disturbance to soil during

construction (personal communication). AMDV is a member of the *Parvoviridae* family,

which is known for its resiliency (Barker and Parrish, 2008). As an example, AMDV

antigen titers were not affected (as measured by counterimmuno-electrophoresis) after

a storage period of 180 days at 5°C and 22°C (Cho and Ingram, 1974). AMDV infectivity

was not affected by storage at 56°C for 30 minutes, while temperatures of 80°C for 30

minutes did inactivate the virus (Eklund et al., 1968).

To the best of my knowledge there is currently no published literature on the detection

of AMDV in environmental water samples. Many viruses, other than AMDV, have been

identified in a wide range of water types (Katayama et al., 2002; Haramoto et al., 2005;

Fuhrman et al., 2005; Prado et al., 2011). Due to high dilution of virus particles, water

samples frequently require viral capture and concentration prior to detection (Fong and

Lipp, 2005). Adsorption-elution (AE) filtration is a common viral capture technique.

During filtration viruses adhere to charged filters, this is followed by manipulation of pH

to elute viruses into smaller volumes for subsequent detection. This AE method has

been used for the identification of many viruses, including Hepatitis A, astrovirus,

norovirus, rotavirus, and adenoviruses, in a wide variety of water samples, including

tidal streams, tap, mineral, sea, river, surface, well and waste water (De Paula et al.,

2007; Victoria et al., 2009; Verheyen et al., 2009; Prado et al., 2011).

As reported in Chapter 3, the 1MDS filter method (USEPA, 1996), tended to outperform

the $MgCl_2$ (Katayama et al., 2002) and the $AlCl_3$ (Haramoto et al., 2004) filter methods

for the concentration of AMDV in spiked samples. In this experiment, the 1MDS method

was used to test 27 water samples collected from the Carlton and Meteghan River

watersheds (Digby and Yarmouth Counties) and two ranches (Digby County) for AMDV.

4.2 MATERIALS AND METHODS

4.2.1 Watershed Sampling

On May 25th, 2012, 2 L samples were collected in triplicate from 16 sites located in the

Carlton River and Meteghan River watersheds (WS samples). Sampling locations

included 7 lake, 7 river and 2 creek sites (Figure 4.1). These watersheds were selected

for sampling due to possible and speculated mink ranch runoff (Brylinsky, 2011). All

samples were collected in high density polyethylene (HDPE) plastic sample bottles which

had been rinsed once with the source water (Scotia Plastics, Shubenacadie, NS), at a

depth of approximately 20 cm below the surface. When the water source had a width

greater than two metres, samples were collected at least one metre from the shore. At

each sampling site, conductivity, salinity, dissolved oxygen, pH and water and air

temperature were measured. Conductivity and salinity were measured using a YSI 85
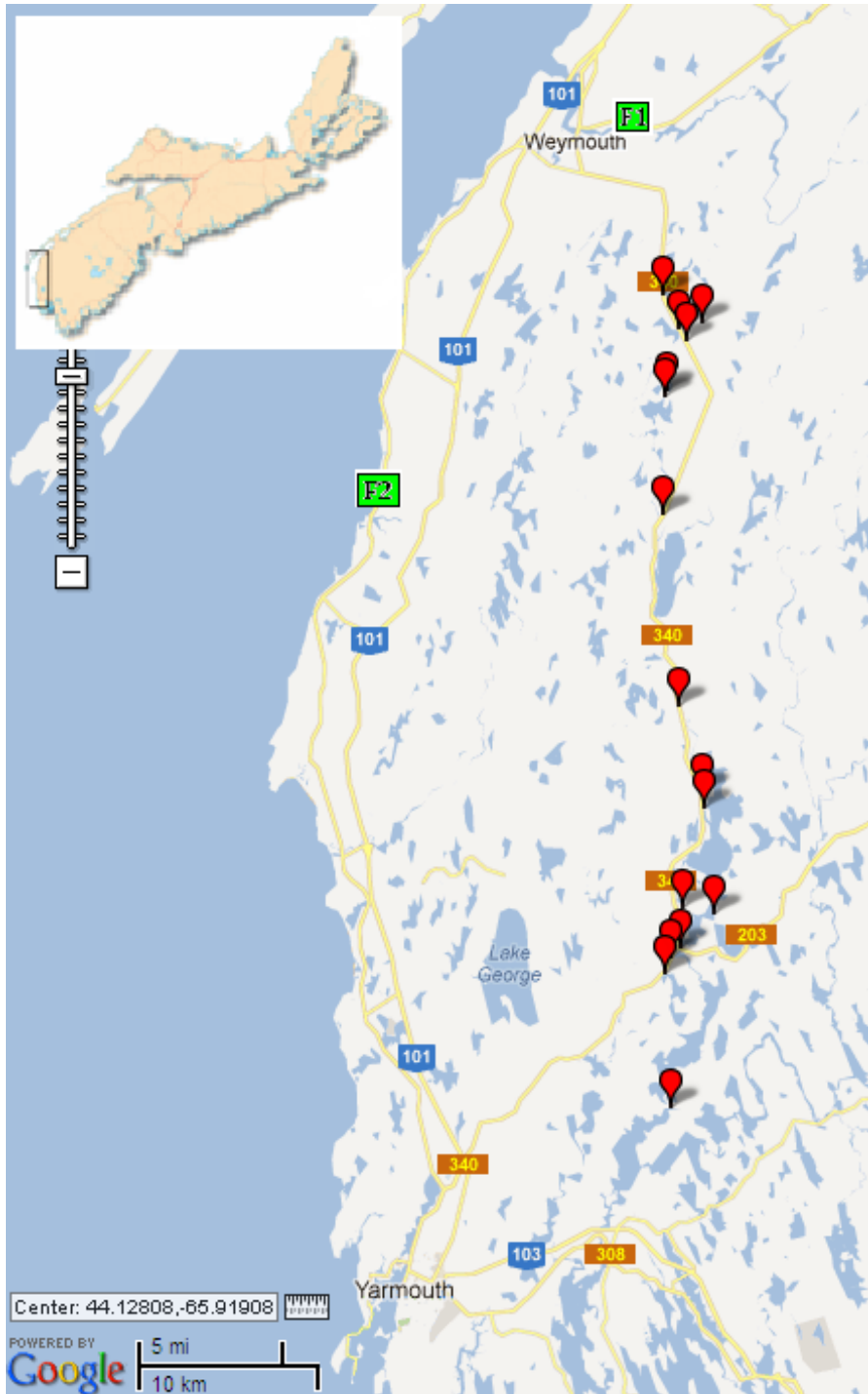
Figure 4.1 Map Indicating Locations of Farm and Watershed Sampling Sites. Inset indicates region of NS where samples were collected. Red points indicate watershed sampling sites and green boxes indicate farm sampling sites.

Oxygen, Conductivity Salinity + Temperature meter (YSI 85) (Fisher Scientific), as explained by De Paula et al. (2007). The OxyGuard Handy Polaris meter (OxyGuard) (Point Four Systems, Coquitlam, BC) was used to measure dissolved oxygen. Water temperature readings were taken using both the YSI 85 and the OxyGuard meters and were reported as the average of the two readings. Air temperature was measured using only the YSI 85 and pH readings were taken using a portable pH meter (The Data Logger Store, Contoocook, NH). After transport, samples were stored at 4 °C until filtration.

4.2.2 Farm Sampling

On July 5, 2012, at a total of 11 sites located on the Heritage and Sabine Fur Farms (Figure 4.1), 2 L samples representing both inflow and outflow water were collected in triplicate, in HDPE plastic bottles (F samples). Both ranches were identified by the owners as AMDV infected. At the Sabine Farm (farm 1), samples were collected by the researcher or the owner. Samples from the Heritage Farm (farm 2) were collected by ranch employees. At each farm, after all samples were collected, the conductivity, salinity, dissolved oxygen, pH and water temperature were measured, using the YSI 85, the OxyGuard and the pH meter, as indicated in Section 4.2.1. After transport, samples were stored at 4 °C until filtration.

4.2.3 Virus Concentration by 1MDS Absorption-Elution Filtration

The 1MDS filtration method, as described in Chapter 3, was used for the filtration of all samples. Briefly, samples were filtered through a 1MDS filter and then eluted with 3 mL plus 2 mL beef extract (1.5% w/v, 0.05 M glycine pH 9.5). The USEPA (1996)

recommends a pre-filter step for samples with turbidity greater than 75 nephelometric turbidity units (NTU).

In this experiment, NTU was not measured, so in cases where filter clogging occurred, filtration recommenced with the addition of a pre-filtration step. In place of the 10 μm polypropylene pre-filter cartridge, outlined by the USEPA (1996), pre-filtration was performed using a Whatman 54, followed by a Whatman 1 and finally, a Whatman 44 (Fisher Scientific) filter housed separately within a Buchner funnel. These filters correspond to removal of particles 22, 11 and 3 μm in size. The neutral charge and the large pore size of these filters allow virus particles to pass through, while retaining larger particulate that were clogging the 1MDS filter. As explained in Chapter 3, secondary concentration was conducted using an Amicon Ultra-4 filter and final volumes were adjusted to 200 μL with Millipore water.

4.2.4 DNA Extraction

All DNA extractions were carried out using the Dynabeads Silane Viral Extraction Kit (Invitrogen) following the protocol recommended by the manufacturer protocol, as outlined in Chapter 2.

4.2.5 Nucleic Acid Concentration and Purity Assessment by Spectrophotometer and Gel Electrophoresis

Prior to PCR amplification, the total nucleic acid (NA) concentrations and purity of both watershed and farm samples were assessed by spectrophotometer (NanoDrop 1000, Fisher Scientific). The concentrations were measured in triplicate and reported as the

average. Purity and contamination was reported as the ratio of absorbance at 260 nm
to 280 nm and the ratio of absorbance at 260 nm to 230 nm. Low 260/280 ratios
indicate protein contamination and low 260/230 ratios indicate contamination by salts or
solvents. The purity of nucleic acids in select samples was also visualized on a 1%
agarose gel. Five µL of sample was mixed with 1.2 µL 5x loading dye. Both samples and
ladders were run for 1 hr at 100V and visualized under UV light.

4.2.6 AMDV Detection by Standard and qPCR

Viral DNA amplifications by standard PCR and quantification qPCR were conducted, as
explained in Chapter 2. In addition to using 1.5, 2.5 and 3.5 µL of sample DNA, samples
that showed very faint amplification were also tested with an additional 4 µL of sample
DNA. Samples that showed streaking were also tested with 2.5 µL of 1:1 and 1:10
dilutions of sample DNA. For qPCR amplifications, due to large variation in DNA
concentrations between samples, and because the source of DNA (viral, genomic or
bacterial) was unknown, additional volumes of extracted DNA (3 µL and 2 µL of 1:10
dilution) were used for samples that had shown faint bands or streaks in standard PCR.

4.2.7 Experimental Design

One litre of the first replicate for watershed samples and one litre of both the first and
second replicate for farm samples, were filtered using the 1MDS filter method. All
standard PCR amplifications were conducted at least twice. Due to time constraints
qPCR was conducted only for farm samples (F1-11). Each sample replicate was tested in
triplicate on two plates. Data were manually edited, as previously reported in Chapter

3. Briefly, if after removal of one replicate the sample had a standard deviation above 0.35, it was deleted. Samples with Ct values higher than the negative control (amplified after the negative) were considered negative. Finally, analysis of the melt curve was conducted to determine if non-specific amplification (double peaks) occurred. For samples above the limit of quantification (LOQ), the amount of virus copies, per mL in the original sample, was calculated, using the equation:

Copies per mL of = copies per µL PCR sample*dilution factor 10 or 1*eluted volume 100
original sample   sample volume filtered

4.2.8 Statistical Analysis

The characteristics of WS and F samples were compared using a 2 sample t-test.

4.3 RESULTS

4.3.1 Characteristics of Water Samples

The water temperature, air temperature, pH, salinity, conductivity and dissolved oxygen for each watershed sample, measured at the time of collection, are shown in Table 4.1. The water temperature for the 16 WS samples ranged between 17.0 °C (sample WS4, culvert Hwy 340) and 22.7 °C (Sample WS15, Sloan Lake). The pH ranged between 8.69 for Nolwans Lake (WS3) and 6.34 for the inflow to Carlton Lake (WS8). Salinity, which was measured in parts per thousand (ppt), was zero for all samples. The average conductivity for WS samples was 52.3 ± 10.33 µS and ranged from 41.4 to 72.5 µS for Ogden (Sample WS10) and Nolwans Lake (Sample WS3), respectively. Dissolved oxygen

Table 4.1 Characteristics of Watershed Samples

Sample number	Location	GPS Coordinates	Water temp°C	Air temp°C	pH	Conductivity µS	Dissolved oxygen mg/L
WS1	Hourglass Lake	44°19.700 065°56.201	19.9	15	7.52	59.8	7.8
WS2	Hilltown Cross Rd Creek	44°18.895 065°54.569	18.4	n/a	7.82	59.9	7.7
WS3	Nolwans Lake	44°18.703 065°55.578	20.0	16.6	8.69	72.5	9.7
WS4	Culvert Hwy 340	44°18.395 065°55.283	17.0	15.3	7.72	72.2	8.6
WS5	Placides Lake	44°16.882 065°56.051	20.1	17.5	7.65	59.7	7.2
WS6	Porcupine Lake	44°16.715 065°56.118	20.3	17.8	7.71	51.0	8.4
WS7	Wentworth River	44°13.214 065°56.223	20.5	18.8	6.56	55.5	3.2
WS8	Inflow to Carleton Lake	44°07.557 065°55.570	20.7	19.2	6.34	43.7	7.5
WS9	Parr Lake	44°05.035 65°54.598	20.2	17.4	6.61	43.9	8.1
WS10	Inflow to Ogden Lake	44°04.520 065°54.520	19.5	17.7	6.74	41.4	8.3
WS11	Inflow to Fanning Lake	44°01.587 065°55.417	20.5	21.3	7.40	43.2	8.2
WS12	Outlet of Fanning Lake	44°00.383 065°55.479	19.6	20.2	6.56	43.3	8.4

Table 4.1 Continued

Sample number	Location	GPS Coordinates	Water temp°C	Air temp°C	pH	Conductivity µS	Dissolved oxygen mg/L
WS13	Mid Fanning Lake	44°01.387 065°54.066	21.7	19.3	6.82	45.6	8.8
WS14	Ryerson Brooke to Raynards Lake	44°00.118 065°55.906	19.7	21.5	6.85	43.8	6.8
WS15	Sloan Lake	43°59.602 065°56.169	22.7	18.3	7.86	56.4	8.0
WS16	Inflow to Vaughan Lake	43°55.670 65°55.926	19.5	21.7	7.36	45.0	8.6
Mean			20.0	18.5	7.38*	52.3	7.8
Standard Deviation			1.26	2.08	0.65	10.33	1.39
% CV			6.3	11.2	8.8	19.8	17.8

*median. Salinity readings for all samples were 0.

was the lowest in the Wentworth River (Sample WS7), at 3.2 mg/L and the highest in

Nowlans Lake (Sample WS3), at 9.7 mg/L, the average being 7.8 ± 1.39 mg/L.

The pH, salinity, conductivity and dissolved oxygen measurements of the farm samples

(F1-11), were more variable than watershed samples, as indicated by larger standard

deviations and coefficients of variation (Table 4.1, Table 4.2). The average water

temperature for farm samples was 21.1 ± 0.99 °C. The pH was the lowest for the Dug

Well on farm 2 (4.79, Sample F7) and the highest for Runoff from Pens 5-10 (8.10,

Sample F6), with an average pH of 7.3 ± 1.01. Two samples registered salinity readings

(Sample F4, Run off at end of Pen 10, and Sample F7, Dug well) and the average

conductivity was 19.5 ± 52.74 µS. The dissolved oxygen was highly variable, ranging

between 0.2 (Sample F6, Run off from Pen 5-10) and 19.7 mg/L (Sample F11, Pond

lagoon), with an average of 4.9 ± 5.84 mg/L. Water temperature was significantly

different between WS and F samples (p=0.024), pH, conductivity, and dissolved oxygen

were not significantly different (p>0.05).

Sample turbidity values were not directly measured, although turbidity levels did affect

the total volume of sample filtered. Even after pre-filtration, it was not possible for the

full 1 L to be filtered for samples F4, F5, F6, F10 and F11; final filter volumes are

reported in Table 4.3.

Table 4.2 Characteristics of Farm Samples

Sample number	Farm	Location	Water temp°C	pH	Salinity ppt	Conductivity µS	Dissolved oxygen mg/L
F1	1	Hose in shed	19.9	7.45	0	0	5.5
F2	1	Waterline Pen 1	20.2	7.33	0	0	1.8
F3	1	Waterline Pen 12	19.7	7.13	0	0	8.8
F4	1	Run off at end of Pen 10	21.4	7.47	0.5	0.9	0.2
F5	1	Run off at end of Pen 3	21.4	7.42	0	0	0.3
F6	1	Run off from Pens 5-10	21.9	8.10	0	0	0.2
F7	2	Dug well	20.8	4.79	0	3.5	5.1
F8	2	Drilled well	20.2	8.04	0.1	176.6	4.1
F9	2	Pen line	21.7	7.18	0	3.1	8.1
F10	2	Outflow ditch	21.6	6.66	0	28.2	0.4
F11	2	Pond lagoon	22.9	8.77	0	2.2	19.7
Mean			21.1	7.42*	0.1	19.5	4.9
Standard Deviation			0.99	1.01	0.15	52.74	5.84
% CV			4.7	13.6	150.0	270.5	119.2

*median

Table 4.3 Summary of Final Volumes Filtered For Samples Requiring Pre-filtration

Sample number	Volume filtered for first replicate (mL)	Volume filtered for second replicate (mL)
F4	200	410
F5	150	120
F6	450	560
F10	450	410
F11	300	220

4.3.2 Nucleic Acid Evaluation by NanoDrop and Gel Electrophoresis

The concentrations of NAs in watershed and farm samples are reported in Table 4.4 and Table 4.5. The tabulated values represent the total nucleic acids in the sample, not the amount of AMDV DNA. These values only provide an indication of the possibility of PCR amplification. The nucleic acid (NA) concentrations ranged from 7.98±0.16 ng/µL in Sample WS1 and 253.22±2.58 ng/µL in Sample F5a. The 260/280 ratio ranged from 11.74±7.47 in Sample F9a to -12.25±24.85 in Sample F2a. The range for the 260/230 ratio was 0.70±0.03 to 0.01±0.00 in Sample F5a and F2a, respectively. Low 260/280 and 260/230 ratios indicate co-extracted contaminants.

The visual evaluation of extracted NAs for Samples F4, F6, F9, F10, F11 is shown in Figure 4.2. The AMDV negative mink genomic DNA sample has a band larger than the 20000 bp ladder. All samples, excluding the spleen homogenate control, have a similarly sized band. Samples F4, F6, F10, and spleen homogenate had streaking, indicative of NAs of varying sizes.

Table 4.4 DNA Concentration of Watershed Samples Measured by Nanodrop

Sample number	Nucleic acid concentration ng/µL	260/280	260/230
WS1	18.19±0.55	1.71±0.02	0.41±0.01
WS2	16.80±2.08	1.62±0.03	0.31±0.02
WS3	11.97±0.31	1.94±0.25	0.11±0.00
WS4	17.41±0.45	1.57±0.08	0.20±0.01
WS5	8.24±0.68	2.00±0.08	0.16±0.02
WS6	7.98±0.16	1.76±0.08	0.11±0.01
WS7	9.53±0.58	1.84±0.25	0.13±0.01
WS8	10.15±0.70	1.86±0.08	0.13±0.01
WS9	9.19±0.19	1.84±0.11	0.15±0.01
WS10	14.05±0.74	1.70±0.02	0.17±0.01
WS11	12.60±0.58	1.91±0.09	0.21±0.01
WS12	10.18±0.63	1.72±0.04	0.18±0.01
WS13	12.88±0.72	1.93±0.06	0.10±0.01
WS14	16.03±0.11	1.53±0.03	0.10±0.00
WS15	10.16±0.83	1.73±0.13	0.07±0.00
WS16	11.42±0.56	1.69±0.03	0.08±0.00
Neg1	2.37±1.75	1.36±2.63	0.08±0.06
Neg2	5.95±2.10	2.50±0.44	0.05±0.02

All values are the average of 3 measurements

Table 4.5 DNA Concentration of Farm Samples Measured by Nanodrop and the Results of Standard PCR Amplification

Sample number	Ranch #	Nucleic acid concentration ng/µL	260/280	260/230	Standard PCR result	qPCR Result
F1a	1	2.26±0.39	2.82±0.97	0.02±0.01	-	-
F1b		4.63±0.84	4.72±1.80	0.05±0.01		
F2a	1	1.23±0.19	-12.25±24.85	0.01±0.00	-	+
F2b		1.24±0.24	-0.27±2.96	0.01±0.00		
F3a	1	1.44±0.34	-1.13±7.55	0.01±0.01	-	-
F3b		2.99±0.21	6.53±3.48	0.03±0.00		
F4a	1	72.14±1.02	1.66±0.01	0.40±0.01	Streaks in 'a' replicate	+
F4b		71.39±0.92	1.45±0.02	0.36±0.00		
F5a	1	253.22±2.58	1.32±0.00	0.70±0.03	-	+(only b rep)
F5b		53.12±0.25	1.47±0.02	0.36±0.00		
F6a	1	104.95±1.26	1.87±0.02	0.62±0.00	+	+
F6b		40.56±0.29	1.90±0.03	0.33±0.01		
F7a	2	11.92±0.53	1.67±0.17	0.08±0.01	-	-
F7b		14.15±0.66	1.54±0.05	0.11±0.01		
F8a	2	25.69±0.56	2.16±0.02	0.07±0.01	-	-
F8b		0.69±0.29	0.14±1.47	0.01±0.01		
F9a	2	3.12±0.59	11.74±7.47	0.03±0.01	Faint bands in 'a' replicate	-
F9b		1.98±0.41	-5.28±0.89	0.02±0.00		
F10a	2	83.86±0.53	1.90±0.02	0.60±0.01	Streaks in both replicates	-
F10b		95.93±0.57	1.93±0.02	0.71±0.01		
F11a	2	14.06±1.02	2.03±0.12	0.09±0.01	-	+
F11b		12.57±0.72	2.50±0.28	0.10±0.00		

All values are the average of 3 measurements
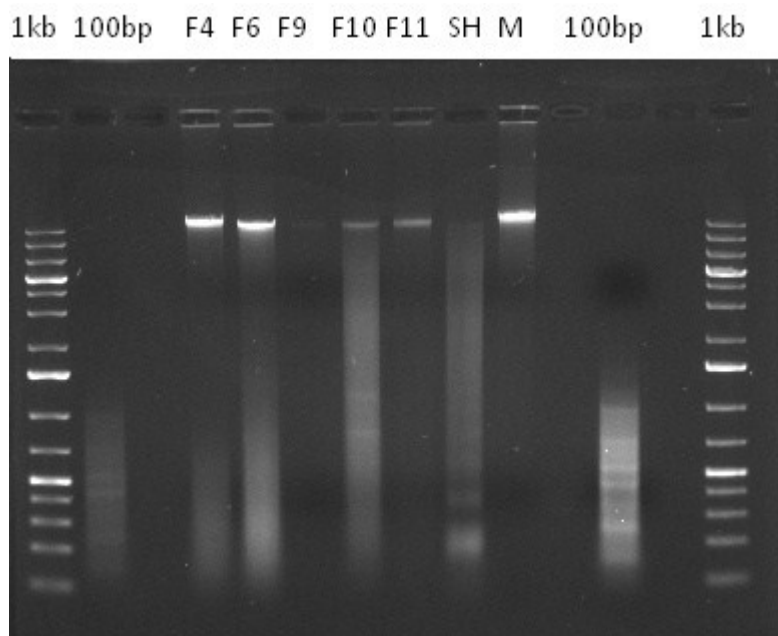
Figure 4.2 Visualization of Extracted Nucleic Acids from Sample F4, F6, F9 and F10 Before PCR Amplification. 1kb indicates ladder, 100bp indicates ladder (degraded) SH indicates spleen homogenate, M indicates AMDV negative mink DNA.

4.3.3 Detection of AMDV by Standard PCR

There was no standard PCR amplification for any of the 17 watershed samples. One of the samples from farm 1, Run off from Pens 5-10 (F6), tested positive by standard PCR (Table 4.5). Three samples, F4, F9 and F10 (Run off at end of Pen 10, Pen line and Outflow ditch) were inconclusive because only very faint bands or streaks were visualized on the gel (Table 4.5, Figure 4.3). It is difficult to identify a relationship between DNA concentration and PCR success with only one positive sample (Table 4.5). It was noted that the positive F6 Sample had high NA concentration (104.95±1.26 and 40.56±0.29 ng/µL) and only amplified at the 1:10 dilution (Table 4.5).
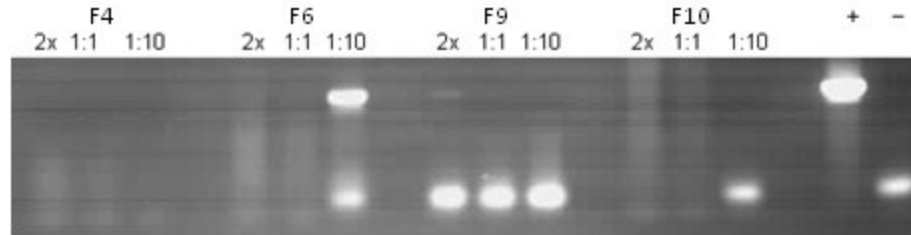
Figure 4.3 Standard PCR of Sample F4, F6, F9 and F10. Each sample was run at three different concentrations, 2x – indicates 2µL undiluted DNA, 1:1 – indicates 2µL of 1:1 dilution and 1:10 – indicates 2 µL of 1:10 dilution.

4.3.4 Detection and Quantification of AMDV in Farm Samples by qPCR

qPCR results were considered positive when amplification occurred before the earliest negative control, and thus sample Ct values were lower than that of the blank. As mentioned in Section 4.2.4 various amounts (3 µL, 2 µL, 2 µL of 1:10 dilution) of DNA were used in PCR reactions. Two µL of the 1:10 dilution (equivalent to 0.2 µL original sample) produced the majority of the positive amplifications (Table 4.6). Samples were considered positive when AMDV was detected on two or more plates. Sample F2, F4, F5, F6 from farm 1 and F11 from farm 2 tested positive (Table 4.6). Sample F5 (Run off at end of Pen 3) had amplification in only one replicate on two plates, this indicates dissimilar replicates which may have contained low viral concentrations, amplification inhibitors or contamination. Detection of AMDV by qPCR produced more positive samples than standard PCR indicating improved sensitivity.

The LOQ was set at 22 viral copies per µL of extracted DNA which was equivalent to the last point on the standard curve. Sample F2 could not be quantified because it was below the LOQ. It is important to note that Sample F11 had a small second peak in the melt curve analysis, between 83 °C and 86 °C (Figure 4.4), but was not removed from

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analysis. As mentioned in Section 2.2.4, such samples cannot be accurately quantified;

due to the additive effects of two peaks, the Ct value is inaccurate. As a result of this

additive effect and because only 2 of the 3 replicates were above the LOQ, Sample F11

was not quantified.

Table 4.6 Summary of Positive AMDV Amplifications by qPCR

Sample	# of positive amplifications (max 4)[d]	Amplification in 'a' and 'b' replicates Yes/no[c]	Equivalent Sample DNA volume per PCR rxn (µL)	Average Ct value ± SD	Copies per µL of sample used for PCR	Copies per mL of original sample
F2	2	Y	3	33.85 ± 0.62	n/a[a]	n/a[a]
F4	3	Y	0.2	24.17 ± 0.33	11380 ± 5475	33471 ± 16102
F5	2	N	0.2	26.44± 0.28	2659 ± 486	22160 ± 4049
F6	4	Y	0.2	24.55 ± 0.30	8477 ± 3356	16787 ± 6646
F11	3	Y	0.2,2 and 3	32.52 ± 0.69	n/a[b]	n/a[b]

[a] Quantification not reliable because values were between LOD (amplification before blank) and LOQ (22 copies/ µL).
[b] Due to additive effects of double peak and close proximity to the LOQ, quantification was not conducted.
[c] Each sample was processed in duplicate, i.e. two 1 L aliquots of sample was filtered.
[d] Replicate samples were run on two plates for a total of 4 possible amplifications.
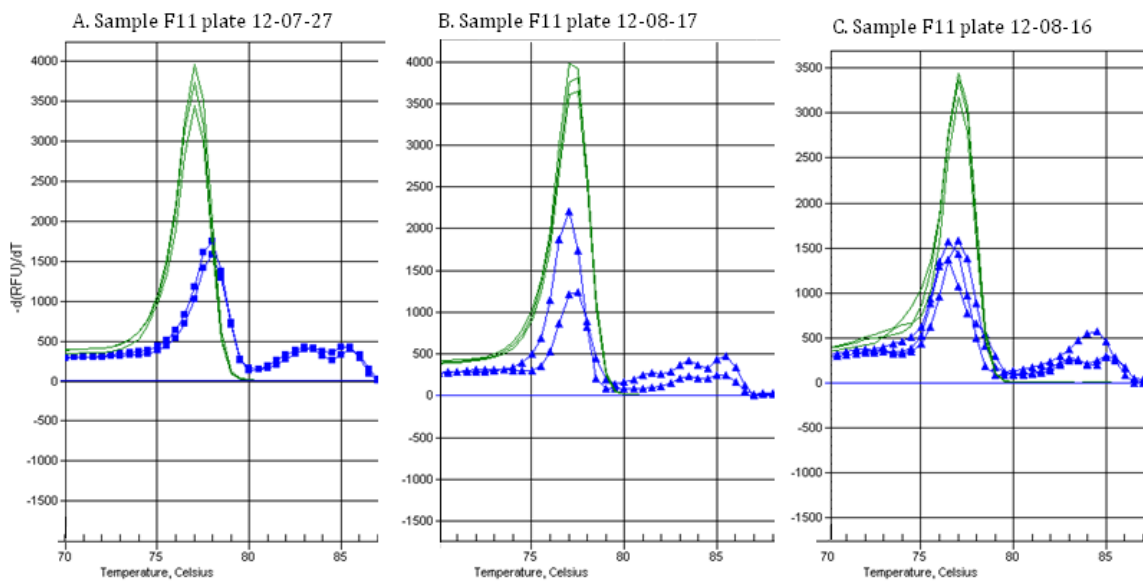
Figure 4.4 Melt Curve Analysis of Sample F11. A, B and C are melt curve results from the 3 qPCR plates on which sample F11 amplified. Green lines represent the last point of standard curve and blue lines represent the sample.

4.4 DISCUSSION

4.4.1 Characteristics of Water Samples

The southwest region of NS that was chosen for sampling is an area with a high concentration of mink farms (Farid et al., 2012). The Carlton River watershed has experienced high nutrient loading, which has been speculated to originate from mink ranch runoff (Brylinsky, 2011; Werring, 2011). The pH of all watershed samples fell between 5 to 9, which includes values that are outside the acceptable range of 6.5 to 8.5 for recreational water (Health Canada, 2012). Nowlans Lake had the highest pH, 8.69, of all watershed samples tested (Table 4.2). The pH measurements of this lake have risen, from 6.5 in 2008 to 7.5 in 2009 and 8.5 in 2010 (Taylor, 2009; Taylor, 2010; Brylinsky, 2011). The pH value of this lake in the current experiment indicates that this

trend is continuing. This may be due to runoff originating from a nearby mink farm

(Brylinsky, 2011). It is important to note the variability in sample pH because pH affects

virus adhesion to filters (Lukasik et al., 2000), therefore changes in pH can affect virus

recovery.   In this experiment, due to the lack of positive samples, pH could not be

correlated to AMDV detection.

Conductivity measurements of the watershed samples, which are related to total

dissolved solids, were within the range previously reported for similar samples (37 to

303 µS) taken within the same Digby and Yarmouth County watersheds, many at the

same locations (Taylor, 2009; Taylor, 2010). Variability in these numbers occurs

naturally due to the surrounding rock and environmental mineral inputs. Dissolved

oxygen levels in this experiment (3.2 to 9.7 mg/L) were also comparable to the range

previously reported for similar watershed samples (4 and 10 mg/L) (Brylinsky, 2011).

Both dissolved solids and dissolved oxygen are speculated to influence the survival of

viruses in water sources (Fong and Lipp, 2005). Again, due to limited positive samples in

this experiment, it was not possible to correlate AMDV detection to these

characteristics.

The pH, salinity, conductivity and dissolved oxygen of farm samples were more variable

than watershed samples, as indicated by the larger coefficients of variation (Table 4.1,

Table 4.2). This higher variability is logical, because the watershed system is one inter-

related body of water, while each ranch is an isolated system with different origins of

water. The variability among ranches suggests that water characteristics are specific to

each ranch and the results of any experiment, on any specific ranch, cannot be extrapolated to other ranches in the region.

Both farms apply lime to manure droppings below cages to decrease smell and to act as a disinfectant. This treatment may be the reason for the higher pH values observed in runoff samples (F4, F5, and F6) and the Pond lagoon (F11). In the past, caustic soda had also been used, which may have some residual effect on ground water. Farm 2 adds bleach to the drilled well as a precautionary measure against pathogens. This procedure had most recently been conducted the day before sampling. Bleach may account for the higher than average pH and conductivity values in Sample F8 (Drilled well). Also, the breakdown of bleach produces salt (NaCl) (Lister, 1956), this could account for the salinity value of Sample F8. Sample F11 (Pond lagoon, farm 2) had a very high dissolved oxygen concentration. It is speculated that algae present in this sample, through photosynthesis, were responsible for the high dissolved oxygen. Algae were also the speculated cause of the green sample colour.

4.4.2 Evaluation of Sample Nucleic Acids

In theory, only one copy of target DNA is required for successful PCR amplification. In practice, approximately 10^4 copies of target DNA per reaction is recommended (New England Biolabs Inc.). In this experiment, it was not possible to measure the amount of target DNA because viral DNA could not be distinguished from other types of NAs.

Aside from target DNA concentration, successful PCR amplification is dependent on sample purity. A 260/280 ratio of approximately 1.8 is indicative of DNA free of protein

contamination (Thermo Fisher Scientific, 2011). All samples that amplified, except F2, had 260/280 ratios between 1.45 and 2.5 (Table 4.5), indicating the presence of some impurities in the samples. Sample F2 had a very low 260/280 ratio of -12.25 ± 24.85, yet the sample was amplified. The impurities were not PCR inhibitors. The deviation of 260/280 ratios observed in this experiment were caused by co-extraction of contaminates that absorb at or below 280 nm, such as proteins and phenols. Low NA concentrations (>10 ng/µL or less) may have also been a factor for large deviation from the expected value of 1.8 (Thermo Fisher Scientific, 2011).

The 260/230 ratio is a second measure of NA purity. This ratio is often higher than the 260/280 ratio for pure NA samples (Thermo Fisher Scientific, 2011). The 260/230 ratios of all samples in this experiment were low and ranged between 0.01 ± 0.00 for Sample F2a and 0.71 ± 0.01 for Sample F10b (Table 4.4, Table 4.5). Low 260/230 ratios are a result of contaminants that absorb at 230 nm, such as organic compounds, salts and urea (OGT, 2011). It is speculated that the low 260/230 ratios in this experiment were caused by the low NA concentrations, the presence of organic matter in watershed samples and the presence of urea and organic materials in farm samples. The results clearly suggest that obtaining high quality DNA from natural water samples and farm water samples is a challenge, which may influence the outcome of PCR amplification.

Visualization of NAs in Samples F4, F6, F9, F10 and F11 (Figure 4.2) indicated that all samples contained NAs larger in size than $2x10^4$ kb. Samples F4, F6, F9, F10, and F11 had a band similar in size to the mink genomic DNA that was used as the control. The

presence of such a band indicates large DNA fragments. The origin of this DNA may be

mammalian, but DNA from bacteria, fungi and other viruses are also suspected to

contribute to this band, as they have been reported in abundance in many other water

samples (Martini, 1992; Whitman et al., 1998).   The majority of this DNA would have

originated from small negatively charged cells and microorganisms (less than 0.45 μm)

that were able to pass though the filter. Streaking observed in Samples F4, F6 and F10

indicates that NAs of varying size were present. Brightness in the wells of Samples F4,

F6 and F11 represent proteins, which further indicate that the 260/280 ratios were low,

due to protein contamination.

4.4.3 Detection of AMDV in Watershed Samples by Standard PCR

Considering the poor amplification results of the spiked artificial river water in Chapter

3, the lack of amplification in watershed samples cannot clearly be interpreted as the

absence of AMDV. These samples could have possibly contained virus particles that

were not captured by the 1MDS filter method, or it is possible that samples contained

high concentrations of PCR inhibitors that impeded detection. These samples were not

diluted prior to PCR because they already contained low concentrations of total NAs

(Table 4.5). In future work, the addition of an internal control could help to establish

whether natural water samples contained PCR inhibitors (see Section 4.4.8).

Although the negative results of the watershed samples are refutable, there are many

reasons why they may be correct. The dilution effect of a large volume of water (e.g. a

lake) had a significant impact and watershed samples had NA concentrations below 20

ng/μL (Table 4.4). Although viral detection has been conducted with sample volumes as low as 0.5 L (Haramoto et al., 2005; Haramoto et al., 2009), larger sample sizes could have led to higher amounts of recovered viruses and positive PCR detection. The 1 L sample volume, as used in this experiment, was selected due to the limitations of the filter apparatus and work space within the fume hood of the level 2 bio-containment laboratory where this work was conducted. The lack of amplification could also have been due to poor sample quality, as indicated by the low 230/280 ratios.

Bacteria use capsid proteins of viruses as a substrate for growth (Bitton, 1980). Bacteria in the watershed samples may have degraded the protective protein coat of AMDV particles. Predation of virus particles by bacteria may thus have also been a factor contributing to the absence of AMDV in watershed samples. Further work and qPCR is required to determine, with a high degree of certainty, that these watershed samples are AMDV free.

4.4.4 Detection of AMDV in Farm Samples by Standard PCR

F6 (Run off from pen 5-10, farm 1) was the only sample to test positive for AMDV by standard PCR (Figure 4.2, Table 4.5). The two replicates of this sample had NA concentrations of 104.95±1.26 ng/μL and 40.56±0.29 ng/μL and 260/280 ratios of 1.87 and 1.90. PCR success cannot be entirely related to NA concentration because of the diversity between replicates and the similarity to other sample concentrations. This sample only amplified when 2 μL of 1:10 diluted DNA was used. Failure of the PCR

amplification for the undiluted samples indicates the possible presence of inhibitors, high concentrations of DNA or high concentrations of Mg^{2+}.

The faint band in Sample F9 (Pen line, farm 2) may have resulted from a low viral DNA concentration, as the total NA concentration was low (3.12±0.59 and 1.98±0.41ng/µL) (Table 4.5). Without further investigation, it is not possible to pinpoint the cause of streaks observed in Sample F4 (Run off at end of pen 10, farm 1) and F10 (outflow ditch, farm 2). The smeared bands are most likely caused by non-optimal PCR conditions. In this experiment, the most probable factors contributing to smears were either an incorrect template to taq polymerase enzyme ratio or non-optimal amounts of Mg^{2+} (Roche; Pestana et al., 2010). In this experiment, AMDV DNA concentrations were unknown and, as such, it was impossible to determine if the target was at the appropriate concentration of 10^4-10^6 copies/rxn (Roche; Pestana et al., 2010). A low concentration of magnesium chloride will result in inefficient PCR amplification, while too high a concentration will lead to non-specific products being formed (Pestana et al., 2010). As samples were suspected to contain magnesium, it is probable that Mg^{2+} concentrations may have been high in the PCR reactions. In future work, magnesium concentrations in natural water samples should be determined prior to PCR.

4.4.5 Detection of AMDV in Farm Samples by qPCR

Due to lack of time watershed samples were not tested by qPCR. The three volumes of DNA (3 µL, 2 µL of undiluted and 2 µL of 1:10 dilution) that were used in qPCR amplification reactions for farm samples provided different results. Amplifications were

most successful when 2 µL of 1:10 diluted DNA was used (Table 4.6). The Ct values for

Samples F2, F4, F5, F6 and F11 were lower than the Ct of the negative control and were

thus, considered positive (Table 4.6). Three of these samples (F4, Run off at end of Pen

10, F5, Run off at end of Pen 3, and F6, Run off from Pens 5-10) only amplified after 1:10

dilution of DNA, which indicates non-optimal PCR conditions, such as the presence of

inhibitors, high Mg^{2+} or high DNA concentrations in the non-diluted samples.

Amplification in only one of the two replicates of Sample F5 may suggest inconsistencies

when sampling, or contamination during processing. Inconsistencies during the

sampling process are the suspected cause of the variability between replicates of this

sample. This sample was collected from a shallow water source with a low volume of

water and the sampling bottle touched the bottom; therefore, it is possible that

sediment was stirred up during collection. Contamination was not identified in any

samples from other sites and so it would seem that the laboratory protocols were

properly followed.   In Sample F2 (water line for pen 1, farm 1), AMDV was detected by

qPCR, but at a level too low to quantify (below the LOQ of 22 copies/ µL of concentrated

sample). The NA concentrations of this sample were very low (1.23 ± 0.19 and 1.24 ±

0.24ng/µL) (Table 4.5) and only amplified with increased (3 µL) DNA volume on only 2 of

the 4 plates (Table 4.6). AMDV was detected only in this water line and not in the line

for Pen 12 (Sample 19) or in the water source (Sample F1). It is difficult to speculate

how contamination is contained to this line and not the source, but a dirty faucet at the

end of the line could contaminate a sample as it flows out. This result is of considerable

concern because it indicates that mink housed in Pen 1 could be exposed to AMDV

contaminated water. Sample F6 (Run-off from Pens 5-10), was the only sample that

amplified in all 4 reactions. This sample was the only farm sample that was collected

outside the ranch fence and identifies a potential source for infection of wild animals.

Although AMDV was not detected in any of the samples on farm 2, it was detected in

the Pond lagoon (Sample F11). This man-made lagoon was the final destination of all

ranch waste water. The presence of AMDV in the lagoon, but not in other samples from

this farm, may indicate that the virus originated from a source which was not sampled.

It is also possible that water characteristics changed within the lagoon to produced

samples more compatible for viral filtration and detection, such as the dilution of

inhibitors. qPCR success cannot be related to specific NA concentrations because

positive results were obtained across a wide range (Table 4.5).

4.4.6 Quantification of AMDV in Farm Samples by qPCR

Using an LOQ equivalent to the last point on the standard curve (22 copies/µL), 3

samples were at or above the LOQ and were quantified. Samples F4, F5 and F6 had

concentrations of $3.35 \pm 1.61 \times 10^{4}$, $2.22 \pm 0.40 \times 10^{4}$ and $1.68 \pm 0.66 \times 10^{4}$ viral copies/mL

of original sample, respectively. These values were calculated assuming 100% recovery

for each step in the concentration process (filtration, secondary concentration and DNA

extraction) because 100% recovery is unlikely these values are likely under

representations. Jiang et al. (2001) reported human adenovirus concentrations of 7.5

genomes/mL in river water samples, Kasorndorkbua et al. (2005) reported a

concentration of 16.7 HEV genome equivalents/mL in swine manure storage samples

and Prado et al. (2011) reported concentrations of 1.2x10^5 human NoV genome copies/mL in treated hospital effluents. The concentrations in this experiment are comparable to those reported by Prado et al. (2011). The differences between high and low concentrations can be attributed to the uniqueness of each experiment, including the capturing and PCR quantification methods used. Jiang et al. (2001) and Kasorndorkbua et al. (2005) estimated concentrations based on standard PCR results, which is inherently inaccurate. Prado et al. (2011) measured concentrations using qPCR. Another major difference amongst the above experiments was the filtration method employed. Jiang et al. (2001) used the vortex flow filtration method, while Kasorndorkbua et al. (2005) used ultracentrifugation and Prado et al. (2011) used adsorption-elution filtration. The virus concentrations obtained in the current experiment are comparable with the results of Prado et al. (2001) and may be due to similar quantification and filtration methods.

4.4.7 Sensitivity of Standard PCR versus qPCR

In this experiment, the ability to detect AMDV varied with the assay method used. With standard PCR, only 1 of 11 farm samples tested positive and 3 samples were inconclusive. When tested by qPCR, 5 of 11 samples were positive. Although unlikely in this experiment, qPCR can produce false positive results when a cut-off value is set too high (Burns and Valdivia, 2008). This is not suspected as the melt curve analysis indicates that the amplified target was the appropriate length and contained the primer sequences. Only one of the inconclusive standard PCR results was positive by qPCR. It is difficult to determine why 2 samples were inconclusive by standard PCR and negative by

qPCR. The results indicate that qPCR is more sensitive and better suited for detection of AMDV in environmental samples. This finding, also discussed in Chapter 2, agrees with the literature that qPCR is a more appropriate detection method (Locatelli et al., 2000) and sensitive detection method (Dagher et al., 2004; Nagaraj et al., 2006).

4.4.8 Weakness of the Filter and DNA Extraction Methods

The 1MDS filter method in conjunction with the Dynabeads extraction kit, used in this experiment, was successful for the detection of AMDV in field samples. However, as mentioned in Section 4.4.3, this 1MDS/Dynabeads produced unclear negative results, as the cause, whether due to lack of target DNA, the presence of inhibitors or non-optimal PCR conditions was unknown. Ambiguous results are an issue for any PCR amplification but are especially important for environmental samples that contain low amounts of target DNA and unknown types and amounts of inhibitors. To mitigate this issue, future work should include internal controls to identify the cause of the negative results.

An internal amplification control (IAC) differs from an external positive control in that it is co-amplified in the same tube as the target sequence. An IAC allows for the detection of amplification inhibition, an outcome that is not possible with an external control (Hoorfar et al., 2004), such as was used in this experiment. IACs can be divided into two categories: competitive and non-competitive. With competitive IACs, the primers are identical to those of the target but the IAC DNA template length is altered. Non-competitive IACs use unique primers and the IAC DNA template is a different sequence, compared to that of the target. Both competitive and non-competitive IACs

have been used with RNA and DNA amplifications (Hoorfar et al., 2004; Hyeon et al., 2010; Deer et al., 2010).

Competitive IACs have been previously used to detect inhibition during detection of norovirus and hepatitis A in shellfish tissue (Atmar et al., 1995) and caliciviruses in water samples (Huang et al., 2000). The absence of IAC amplification indicated PCR inhibition. Huang et al. (2000) reported competitive amplification effects between the IAC and the target. Decreasing the IAC concentration and increasing the IAC length can help to alleviate such competition (Hoorfar et al., 2004). While this method identifies PCR inhibitors, it does not give any indication of the presence or the effects of filter inhibitors.

In order to measure inhibition at filtration, Fuhrman et al. (2005) cut filters in half after filtering field samples. One side was spiked with a known amount of poliovirus, which was then extracted and amplified by PCR. Spiked filter samples were compared to a sample in which poliovirus was added directly to PCR reaction. The difference between the two gave an indication of amplification inhibition. Fuhrman et al. (2005) reported concentration-dependent viral recovery rates in their spiking experiments. When higher amounts of virus were added (up to 330,000 enterovirus particles/mL), recovery rates were higher (51.2±1.9%), compared to recovery rates (16.7±0.8%) for lower amounts of virus (6.6 to 6,600 enterovirus particles/mL). As a result, Fuhrman et al. (2005) suggested that careful consideration be taken when determining the concentration of the control added to measure inhibition, because both very large and very small amounts may affect recovery rates. Regardless of the obvious increase in time and cost,

inclusion of internal controls will provide more reliable negative results. Future work

should include, at a minimum, a competitive IAC. The construction of IACs involves

modification (shortening) of the target PCR products and cloning in a plasmid. The

details of IAC construction are out of the scope of this document and are described

elsewhere (Abdulmawjood et al., 2002; Hoorfar et al., 2004).

4.4.9 Conclusion

This is the first reported experiment in which AMDV has been detected in non-animal

waste or tissue samples. Sites containing AMDV and thus potential sources of infection

for both farmed and wild animals were identified. This experiment demonstrates that

the 1MDS filter method in conjunction with secondary concentration, DNA extraction

and assay by PCR, is capable of detecting AMDV from mink ranch water samples. It is

unclear whether the negative results obtained for both watershed and ranch samples

were legitimate or whether they were false negatives, due to inhibition. Further work,

with the addition of internal controls to detect PCR and filtration inhibition, would

greatly improve this method. This work should lead to better virus control, increased

knowledge of virus movement and improved management of AMDV.

CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

The first conclusion of this research is that the DNA extraction kit employed affects the sensitivity of AMDV detection by PCR. Under the conditions tested, the DB and QI kits outperformed the ZR and AX kits. This result indicates the importance of using a properly tested DNA extraction kit to reduce the possibly false-negative results and increase detection sensitivity. Although the DB extraction kit was used throughout this study, it is recommended that the QI kit be considered as a replacement for the DB due to its lower cost and shorter procedure duration.

Secondly, the adsorption-elution method is capable of concentrating AMDV from spiked samples. The 1MDS method outperformed or was comparable with the $MgCl_2$ method and both outperformed the $AlCl_3$ method. Virus recovery was highly variable and water impurities are the speculated cause of low and variable recovery. Further optimization of the method is required with specific attention to sample pH and ionic concentrations. Also, scale-up of the filtration apparatus would allow for processing of larger sample volumes and thus improve the chance of capturing virus when sample concentrations are very low. Identification of impurities common to environmental samples and that impede filtration or detection would be of great benefit.

Thirdly, the 1MDS method was successful for detection of AMDV in 5 of 11 water samples collected from 2 NS mink ranches. These sample sites are possible sources of ranch re-infection or virus transmission to wild animals. The cause of negative results, whether due to low viral copy number or the presence of PCR inhibitors remains

unclear. The low success rate of recovering AMDV from artificial river water suggests that this is perhaps not a suitable sample matrix and real river water should be used in future concentration testing. It is important that the investigation into the causes of negative PCR results be continued. The inclusion of internal amplification controls may lead to the reduction of possible false negative results and will thus add substantial confidence to the results. Overall, the implementation of this method (1MDS filtration and PCR testing) on more ranch and environmental samples will lead to an improved understanding of virus transmission and will lead to enhanced virus control strategies. Increased development of virus control approaches will result in the reduction of virus entry onto ranches, thus, a decrease in animal infection is possible.

APPENDIX A. RAW DATA SUMMARY CHAPTER 3 TRIAL 1 – COMPARISON OF THREE FILTER METHODS USING MILLIPORE WATER

		1000µl			100µl			10µl			5µl		
		rep	+/total	%	Rep	+/total	%	rep	+/total	%	rep	+/total	%
	1MDS	2	6/6	100.0	3	9/9	100.0	3	7/9	77.8	3	9/9	100.0
millipore	$MgCl_2$	2	6/6	100.0	3	8.5/9	94.4	3	6/9	66.7	3	8.5/9	94.4
	$AlCl_3$	2	5.5/6	91.7	2	6/6	100.0	3	8.5/9	94.4	6	3.5/27	13.0

1µl			0.1µl			0.01µl			0.005µl			0.001µl		
+/total	%	rep	+/total	%	rep	+/total	%	rep	+/total	%	rep	+/total	%
6.5/9	72.2	3	5.5/9	61.1	3	8/9	88.9	3	3.5/18	19.4	3	0.00	0.0
9/9	100.0	3	7/9	77.8	3	1/18	5.6	3	0/9	0.0	3	0.00	0.0
2.5/9	27.8	3	0.5/12	4.2	0			0			0		

APPENDIX B. RAW DATA SUMMARY CHAPTER 3 TRIAL 2 – COMPARISON OF THREE FILTER METHODS USING TAP, WELL AND RIVER WATER

		10 µl			5 µl			1 µl			0.1 µl		
		reps	+/total	%	reps	+/total	%	reps	+/total	%	reps	+/total	%
tap	1MDS	3	10/18	55.6	3	5/9	55.6	6	3.5/21	16.7	3	0/9	0
	MgCl$_2$	6	11/27	40.7	3	6/18	33.3	6	13/18	72.2	3	2.5/9	27.8
	AlCl$_3$	2	0/6	0	2	0/12	0	3	2.5/21	11.9			
river	1MDS	3	7/18	38.9	3	2/15	13.3	3	2.5/12	20.8	2	0/9	0
	MgCl$_2$	3	0/18	0	3	0/12	0	3	0/9	0	2	0/9	0
	AlCl$_3$	3	0/18	0	3	0/9	0	3	0/9	0			
well	1MDS	3	9/9	100	3	10.5/18	58.3	6	12.5/36	34.7	3	0/18	0
	MgCl$_2$	3	7/9	77.8	6	2.5/27	9.26	6	15/27	55.6	3	0/18	0
	AlCl$_3$							3	0/9	0			

APPENDIX C. PROTOCOL FOR OFF-LINE EDITING OF QPCR RESULTS

1. Open data file with Bio-Rad iQ5 software.
2. Highlight only wells with standards and negative controls.
3. Make note E and R^2 values.
4. Evaluate standard deviation for each standard, if necessary remove one of the three reps to obtain standard deviation (sd) below 0.350 and improved E and R^2 values. If 0.350 is not obtained (not as stringent for high Ct standards) the standard must be removed.
5. Organize by Ct mean to check negative controls in relation to last standard
6. Once acceptable sd, E and R^2 values are achieved analyze wells containing unknown samples.
7. Edit as needed to achieve acceptable sd. If an acceptable value cannot be reached the sample should be removed.
8. Export data to excel spreadsheet, samples that amplify after the negative control should be given a value of zero. If samples do not amplify (N/A) in all three reps, or there is high sd at low Ct values they are giving a period (.) indicating that the sample has low confidence and may not have worked.

Condition	Value
High sd and high Ct	0
1 replicate amplified at high Ct, 2 reps N/A	0
High sd at low Ct (2 or 3 reps)	.
N/A all three reps	.

9. In the excel spreadsheet include negative (DNA negative, DNA free), positive and standard controls.
10. Add other necessary factors to spreadsheet (ie. dilution, method etc). If means are to be analyzed individual reps can be removed.

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