Transmission Patterns of Zoonotic and Emerging Pathogens in Canada's North Related to Climate Change

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Abstract

Country food is a term used to describe the traditional food of the Northern Canadian Indigenous population. Country food diets are of great cultural significance but lead to increased risks of contracting zoonotic pathogens as most preparations of meat and fish are uncooked.

Climate change could lead to northward migration or range extension of certain animals, which could result in increased risk of zoonotic transmission in Northern Canada. Hepatitis E virus (HEV) is a zoonotic pathogen that can transmit through the consumption of uncooked meats and causes acute and chronic hepatitis in humans. *Toxoplasma gondii* (*T. gondii*) is a protozoan parasite that causes zoonotic toxoplasmosis in humans. *Toxoplasma gondii* has been observed in Northern Canadian wildlife and can also transmit through the consumption of uncooked meats. This study aims to examine the prevalence of HEV and *T. gondii* in wildlife harvested as country food in relation to climate change.

Caribou, ringed seal and beluga are some of the keystone country food species that were sampled from various Northern Canadian regions. Prevalence was measured using PCR to detect pathogen genomes in serum, blood and tissues. Serological testing was performed using enzyme-linked immunosorbent assays (ELISA) for detection of antibodies against HEV and *T. gondii*.

These methods were successful in detecting both HEV and *T. gondii* in country food samples at higher prevalence than previously reported. There was also detection of HEV in ringed seal (*Pusa hispida)* and beluga (*Delphinapterus leucas*) which is the first time that HEV has been detected in these species. These results suggest that there is a potential food safety risk to humans when consuming country foods in Canada's North. These findings may explain the high seroprevalence of *T. gondii* in humans in these regions.

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1. Introduction

1.1 Foodborne Pathogens

Emerging zoonotic pathogens are of growing concern worldwide due to climate change (Nova et al., 2022). Zoonotic pathogens are the cause of a substantial proportion of human illness worldwide (WHO, 2025). Globally, roughly 60% of all human pathogens and 75% of emerging infectious diseases are zoonotic in origin (Mohammadpour et al., 2020). Many zoonotic pathogens cause infection in humans when tissues are consumed therefore these pathogens are also classified as foodborne pathogens. Foodborne zoonoses is responsible for approximately one third of all zoonotic diseases worldwide (Leahy et al., 2022). In Canada, there are an estimated 4 million cases of foodborne illnesses yearly, with 11,600 hospitalizations and 238 deaths (Public Health Agency of Canada, 2016; Thomas et al., 2013). These were further broken down into various different bacteria, parasites and viruses. The leading cause of foodborne illnesses in Canada is norovirus, with 1.05 million cases per year (65% of all cases) (Public Health Agency of Canada, 2016). The number of cases is most likely underestimated as most often individuals do not seek care after contracting a foodborne illness and laboratory analysis is not conducted to find the culprit of their infection. This also leads to difficulty when trying to link these cases back to the source of the illness as they remain unidentified. According to the CDC (Centers for Disease Control and Prevention), toxoplasmosis is one of the leading causes of death from foodborne illness in the United States (CDC, 2025). From the mid to late 2000s, toxoplasmosis was considered to be the second most lethal foodborne illness only behind *Salmonella* spp. and one of the leading contributors to loss of "quality-adjusted life years" (Batz et al., 2012; Scallan et al., 2011). From 2000 to 2017 there have been 10,432 food products recalled by the Canadian Food Inspection Agency as a result of microbial contamination (Herod et al., 2019). In this review, Herod et al.

stated that there was an increase in food recall events from 87 in the 2000s to 153 from 2010 to 2017.

1.2 Climate Change and Emerging Pathogens

Canadian wildlife are subject to climate change as Canada is warming faster than anywhere else on earth (Séguin, 2008). This has led to an increase of survival and spread of zoonotic pathogens by creating more favorable conditions for host reproduction (Séguin, 2008). Changes in the climate may, therefore, enhance the "host-pathogen cycle" as there could be an increase in host population, an increase in survivability of the pathogen, a larger vector range and potential for host species to become more susceptible to infection (Waits et al., 2018).

With temperatures warming at alarming rates, it is expected that by 2100 there will be a global increase of between 1.0 to 5.7°C (Gao & Cui, 2023). This is of particular importance in the arctic regions because as the temperature rises in these areas severe changes in the weather and precipitation levels will occur. Estimates show there to be a 30% to 60% increase in precipitation due to 1) increased evaporation due to the loss of open sea ice, 2) the increase in moisture transport towards the pole (McCrystall et al., 2021). The increase in precipitation will put higher stress on the water sanitation infrastructure leading to these northern communities with a higher risk of contracting zoonotic pathogens (Waits et al., 2018; Eze et al., 2014).

1.3 Country Food

Country food is a term used to describe the traditional food of the Northern Canadian Indigenous population (Sharma et al., 2013). The Country food diet is extremely intertwined with the local environment (Lemke & Delormier, 2017) and Inuit knowledge of this diet has been paramount to the understanding of local health, environmental and climate sciences in the region (Rapinski et al., 2018; Huntington et al., 2019; Harper et al., 2021). Traditional country food

includes organs and meat from the local fish, birds, and mammals, as well as berries. Country food has a great cultural significance as it is a way to pass down the Inuit *Qaujimajatuqangit* (traditional knowledge), to the next generation of hunters (Allaire et al., 2021). Country food is also important to these communities as it is a way for the younger hunters to learn about their land and the role that each animal plays in their lives (Pearce et al., 2011). According to a 2020 Statistics Canada survey household food insecurity rates in Nunavut, Northwest Territories and Yukon were 57 %, 21.6 % and 16.9% respectively (Canadian Northern Economic Development Agency, 2024). Given the food insecurity rates in Northern Canada, country food has provided these communities with a source of more nutrient dense and sustainable food especially in isolated communities (Brockington et al., 2023; Power, 2008). This traditional diet has been studied for its nutritional value as it is linked to significantly higher intakes of protein and protein-related micronutrients, fibre, calcium, n-3 polyunsaturated fatty acid, riboflavin, niacin, iron, zinc, copper, magnesium, potassium, selenium, and vitamins A, B_6, B_{12}, C, D and E (Dewailly et al., 2001; Egeland et al., 2011; Gagné et al., 2012; Little et al., 2021; Sharma, 2010). Traditional practices of sharing and harvesting country food has also been linked to improved mental health in communities (King & Furgal, 2014; Richmond & Ross, 2009). Climate change has been a large factor in altering the consumption of country foods as migration patterns of animals are shifting (Kenny et al., 2018) and the rapidly changing weather is making the local environments more difficult to navigate or impossible to hunt (e.g., higher water levels and less sea ice) (Beaumier & Ford, 2010; Caughey et al., 2021; Goldhar et al., 2014; Pearce et al., 2012). This increase in temperature has led to an increased risk of exposure to pathogens and toxins found in country food (Harper et al., 2015; Martinez-Levasseur et al., 2020; Nancarrow & Chan, 2010; Parkinson & Butler, 2005). With country food being as crucial as it is to these communities the risk of new zoonotic pathogens must

not be ignored. Many country foods are consumed raw, others are cured, dried, or partially cooked. The butchering of these country foods is done either in the field or in the hunter's homes, where the risk for cross contamination is higher. The tools used to harvest the country food, such as cutting boards and knives, are likely not cleaned between harvesting different tissues or different species leading to a higher likelihood of cross contamination. As a result, consumers are at an increased risk of contracting zoonotic pathogens (Jenkins et al., 2013). Unknown or emerging pathogens in wildlife could cause serious public health risks to northern communities and it is crucial that these communities understand how to reduce the risk of foodborne infection. Routine testing for pathogens by biologists after the hunt would result in an increased knowledge of any new or existing pathogens that potentially could lead to infection.

1.4 Hepatitis E Virus

Yearly, there are approximately 3.3 million symptomatic cases of hepatitis E leading to roughly 44,000 deaths worldwide (WHO, 2022). Most of these cases involve acute infection with a 1 – 4% mortality rate, and with the incubation period lasting two weeks to two months (Guerra et al., 2017). However, acute cases of HEV can become chronic when the individual cannot fight HEV and when the patient presents continuous viremia past three to six months (Ma et al., 2022). This is normally an issue for individuals with compromised immune system, for example individuals with HIV infection, individuals who have undergone organ transplants or people taking immunosuppressive medication (Zhou et al., 2013). There are antivirals that are used in treating both acute and chronic HEV, this includes ribavirin in the acute treatment and, in cases of chronic HEV, a combination of ribavirin and interferon have been used with varying success (Aslan & Balaban, 2020; Zhou et al., 2013). The main symptoms of HEV infection are jaundice, abdominal

tenderness, vomiting, nausea, and fever, and if left untreated the symptoms can worsen to liver failure (Kamar et al., 2012).

HEV has been the subject of concern in sub-tropical countries where sanitation protocols are either more relaxed or non-existent (Kamar et al., 2012). The main mode of transmission is through contaminated water supplies, although sporadic cases of acute HEV have been linked to the consumption of infected meat or direct contact with infected animals (Goens & Perdue, 2004; Meng, 2010). Until recently, it was believed that this virus did not exist in the northern regions of the world however, there is data to suggest HEV is becoming an increasing issue in Europe (Arnaboldi et al., 2021; Sacristán et al., 2021) and in Canada (Borlang et al., 2024).

The seroprevalence of HEV varies from region to region with tropical regions ranging from 27 – 80 % (Guerra et al., 2017) and in non-endemic areas it can vary between 3 – 21 % (Fearon et al., 2017; Wilhelmi et al., 2019). It is also interesting to note that the seroprevalence changes based on the individual's exposure to swine, which is believed to be the main carrier of the virus (Capai et al., 2019).

1.5 Hepatitis E Virus Structure

Hepatitis E virus (HEV) is a quasi-enveloped positive-stranded RNA virus that has a 7.2 kb viral genome (Purdy et al., 2022). Within the genome of HEV there are three distinct open reading frames (ORFs) (Khuroo et al., 2016; Zhou et al., 2013). These ORFs each have a distinct function for the virus. An open reading frame is the region of DNA or RNA that is between the start and stop codon that encodes for proteins. ORF1 has the role of forming non-structural proteins such as, a methyltransferase, a papain-like cysteine protease, a helicase and an RNA-dependent RNA polymerase (Purdy et al., 2022). There is a small junction region between ORF1 and the start site of the subgenomic coding region, therefore ORF2 overlaps with OFR3 but neither overlap

with ORF1 (Cao & Meng, 2012). ORF2 is responsible for the capsid protein formation, of which there are three main subunits (S, P1 and P2) and ORF3 is responsible for the cytoskeleton-associated polyprotein (Cao & Meng, 2012). This polyprotein has the functions of inhibiting the mitochondrial apoptosis pathway (Moin et al., 2007), delaying the transport of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) into the nucleus thereby promoting cell survival contributing to an increase in viral replication (Chandra et al., 2010). Therefore, the protein created by ORF3 is crucial to the creation of a favourable environment for replication and pathogenesis.

1.6 Hepatitis E Virus Classification

HEV is classified under the genus *Orthohepevirus* within the Hepeviridae family, in the *Paslahepevirus* genus (Waqar et al., 2022). There are 8 genotypes of HEV, 5 of which infect humans HEV-1, -2, -3, -4, and -7 (Ahmed & Nasheri, 2023). HEV-3 and -4 are zoonotic and are typically found in industrialized countries and have a broad range of host animals (B. Wang & Meng, 2021b, 2021a). Both HEV-3 and -4 have been linked to the zoonotic transmission of HEV to humans from different animals (Capai et al., 2019). HEV-3 has been known to infect rabbits, swine, and humans in North and South America, Europe, Asia, and Africa and HEV-4 has been known to infect swine, rabbit, deer, rat, and mongoose in regions of Asia and Europe (Roth et al., 2016). HEV-3 is zoonotic and mainly transmitted through the consumption of raw or undercooked meat or close contact with infected animals (Belén Pisano et al., 2020), although it can also be blood-borne (Roth et al., 2016). HEV-4 is also zoonotic and is transmitted through the consumption of undercooked meat (Lhomme et al., 2020). Both genotypes, therefore, represent a public health issue for individuals that are in contact with infected animals or eat raw/undercooked meat and individuals that have compromised immune systems (Lhomme et al., 2020).

1.7 Hepatitis E Virus Transmission

The main transmission route of hepatitis E virus is the fecal-oral route. Generally, hepatitis E virus genotype 1 and 2 in developing countries is spread via water contamination (L. Lu et al., 2006; Teo, 2010), whereas genotypes 3 and 4 are zoonotic in nature and are prominent in industrialized countries. These genotypes mainly spread through the consumption of raw or undercooked meat (P. Li et al., 2020). HEV-5 and 6 were isolated from wild boar samples in Japan (Takahashi et al., 2011). HEV-5 has shown the potential to infect humans as a study done by Li et al. in 2019 has shown that previously seronegative cynomolgus monkeys can be infected with a HEV-5 clone (T. C. Li et al., 2019). HEV-7 has been reported in *Camelus dromedaries* and *Camelus bactrianus*. More recent studies have shown that these genotypes are able to transmit to cynomolgus primates (L. Wang et al., 2019) and to a human liver transplant patient who consumed both camel meat and milk regularly (Lee et al., 2016), therefore the camelid HEV has the potential to zoonotically infect humans.

The pathway of infection of a host begins with the virus entering the body through the gastrointestinal tract where the virus will begin to replicate in the epithelial cells of the intestine before traveling through the bloodstream until it reaches the liver (Williams et al., 2001). The HEV virion is found in both unenveloped and quasi-enveloped forms within the host, both of which have unique entry mechanisms into the cell. There is little known about the viral entry mechanism of the unenveloped HEV, but the quasi-enveloped dynamin-dependent complex process utilizes small GTPases, the Ras-related proteins Rab5 and Rab7, and Niemann-Pick disease type C1-mediated lipid membrane degradation (Yin et al., 2016). Once in the liver the viral RNA of HEV is released into the cell after an unknown uncoating process of the HEV capsid. The ORF1 will be translated into nonstructural polyprotein which encodes for multiple functional enzymes. The viral

RNA dependent RNA polymerase (RdRp) will synthesize a negative sense RNA strand which is used for further viral replication within the cell (Debing et al., 2016; Williams et al., 2001). ORF2 is responsible for producing secreted glycosylated proteins, which forms the viral capsid, to encapsulate the replicated viral RNA into HEV virions (Yin et al., 2018). ORF3 creates proteins that interact with the host cell proteins to create a favorable environment for viral replication and pathogenesis (Gouttenoire et al., 2018). To be released into the body the virions need to fuse with the hepatocyte plasma membrane, where the unenveloped HEV virions are released into the bile duct and the quasi-enveloped HEV virions are released into the bloodstream of the host (Nagashima et al., 2017).

1.8 Hepatitis E Virus Detection Methods

Due to the possibility of emergence in Northern Canada, methods to detect HEV are crucial for epidemiological investigations. Currently there are very few standardized methods for isolating and for detecting HEV.

Commercial ELISA kits are available to detect anti-HEV antibodies. One of the most widely accepted types of kits for veterinary use involves a double-antigen multispecies sandwich to detect the presence of anti-HEV antibodies. These kits are beneficial because they do not require specialized equipment other than a plate reader and they are reported to be highly sensitive in detecting anti-HEV antibodies (Arce et al., 2023 ;Caballero-Gómez et al., 2022). These kits also provide the benefit of having a wide host range, this will allow for testing on a variety of animals that commonly interact with humans.

There are disadvantages that are linked to the use of commercial ELISA kits. The primary disadvantage is a positive result on the test is not guaranteed that the host will have an active infection. Antibody presence in the tested serum may mean the host has encountered the virus but

the active infection stage of the pathogen may be complete. Further testing would be required to tell if the host animal has an active infection (You et al., 2023). This means that the time to collect any further valuable data about the genotype of the virus may be lost. These kits also pose the problem of being relatively expensive when it comes to importing them to developing countries (Arce et al., 2023).

Given the limitations of serological testing the detection of HEV will rely on other methods such as nucleic acid amplification testing such quantitative real-time PCR (qRT-PCR). This technology allows for the use of fluorophores or probes to report the concentration and presence of the desired genetic product in real time (Higuchi et al., 1993; Vautrot & Behm-Ansmant, 2020). The reactions are measured using cycle threshold (C_t) or the time at which the intensity of the fluorophore or probe is greater than the background fluorescence (Jozefczuk & Adjaye, 2011). Therefore, the higher the concentration of desired RNA the faster the fluorescence will break the threshold of the background fluorescence lowering the C_t. There are two key prerequisites to having successful reverse transcription, the first being high quality mRNA and the second being a method of priming the mRNA so that a full-length cDNA strand will form (Nolan et al., 2006). This assay is beneficial as it is able to conduct sensitive and specific quantification of wide variety of sample types in a homogenous format eliminating the need to manipulate and possibly contaminate the samples (Jozefczuk & Adjaye, 2011). There is a drawback to utilizing qRT-PCR as a detection method. Due to the popularity of this assay there are many different protocols created to generate data. This causes a lack of standardization when it comes to each step of the process of conducting qRT-PCR (Bustin, 2005). This calls into question the reproducibility of certain experiments utilizing qRT-PCR.

Droplet digital PCR (ddPCR) is a type of digital PCR that uses oil and water emulsions to form droplets to quantify the amount of nucleic acid within the given sample (Kojabad et al., 2021). These droplets are formed using small microfluidic chips using the prepared sample and the provided droplet oil (Bio-Rad, 2024). The droplet will contain the nucleic material and then PCR amplification will occur. If the droplet contains the desired genetic information, then the TaqMan probe will bind and a fluorescent signal will be emitted (Cecillon & Nasheri, 2024). The detector instrument will measure the amplitude of fluorescence and number of droplets. The software then uses Poisson distribution to calculate the RNA or DNA concentration based on the ratio of fluorescent droplets to nonfluorescent droplets (Kojabad et al., 2021). There are some benefits to utilizing ddPCR over quantitative real-time PCR (qRT-PCR), including a) standard curves are not needed to quantify the viral RNA (Plante et al., 2021; Smith & Osborn, 2009), b) ddPCR utilizes the same primers and probes as the real time qPCR with more reliable and more sensitive results when compared to qRT-PCR (P. Zhu & Wang, 2016), c) The process of generating the droplets and separating the genetic information is beneficial to the quantification of the target as it will reduce the background noise and reduce the inhibition that occurs in bulk PCR reactions leading to more accurate results (Plante et al., 2021; Zhang et al., 2019), and d) its ability to perform absolute target quantification (Plante et al., 2021), while qRT-PCR requires a standard curve to quantify each one of the samples and that standard needs to be precisely measured to ensure accuracy of the results (Dingle et al., 2013; Plante et al., 2021). The main disadvantage of using ddPCR instead of qRT-PCR is the cost. The equipment required for ddPCR is more expensive compared with that for conventional qPCR. Furthermore, operating ddPCR is costly when it comes to the cost of the maintenance contracts, the reagents, and the ancillary materials that are required for the process of droplet generation. (Bio-Rad, n.d.).

1.9 Toxoplasmosis

Toxoplasma gondii (*T. gondii*) is an Amazonian protozoan parasite that has spread worldwide. Studies have shown the high prevalence of the parasite in Northern Canada in a variety of different wildlife species that are harvested as country foods (caribou, seal, etc.) (Reiling & Dixon, 2019). This parasite was originally discovered in 1908 in a rodent species and in a rabbit in Brazil (Innes, 2010). It is estimated that the human seroprevalence of *T. gondii* is around 30%, although this can vary depending on the area of the world and the different exposures to possible hosts (Flegr et al., 2014). It is important to note that most of the infections are asymptomatic, with *T. gondii* lying dormant until reactivated, which only occurs in individuals that have weakened immune systems and newborn/unborn children infected congenitally (Reiling & Dixon, 2019). Upon reactivation, the parasite will replicate from the tissue cysts that are in the organism that is infected (Reiling & Dixon, 2019). The specific issue with this parasite is that the infected newborn humans exhibit no symptoms in most occasions which is problematic because if it is left untreated the parasite can cause irreversible damage to the nervous system and loss of sight (retinochoroiditis) (Robert-Gangneux & Dion, 2020).

Human transmission of this disease is particularly high in areas where the indigenous populations do not cook their meat as part of their traditional diet, leaving the tissue cysts active for infection (Reiling & Dixon, 2019). The seroprevalence of *T. gondii* in humans varies depending on the region of Canada. According to the Public Health Agency of Canada the most recent estimate of seroprevalence of *Toxoplamsa gondii* in Canada was 10.5% (Merks et al., 2024). This estimate was based on serological testing from 2016-2017 within all 10 of the provinces. This estimate however left out the three northern territories. There have been some studies looking at the seroprevalence of *T. gondii* in northern regions Canada. They have concluded that there is a

28% seroprevalence in Nunavut (Egeland, 2010) and a 43% seroprevalence in Nunavik, QC (Ducrocq et al., 2021).

The prevalence of this parasite in Canadian retail meat is an important metric to measure, with pork showing a prevalence of 3.2% (Dubey, 2021). Sheep have also seen a high prevalence of this parasite, with anti-*Toxoplasma* antibodies being present in approximately 9.7% of the tested animals. It is important to note, however, that lambs have a much lower infection rate than the older sheep and the meat that comes from these animals is normally from lambs (Dubey, 2021). Bovine prevalence is lower than the other meat samples at 2.2% (Dubey, 2021) but in a previous study it was noted that there may be inhibition of the proper maturation of the parasite and infection of cattle as they have some sort of innate immunity when it comes to being infected with *T. gondii* (Onyiche & Ademola, 2015). There have been previous reports of high *T. gondii* seroprevalence in arctic wildlife as well. A review article by Reiling and Dixon (2019) reported the prevalence in birds such as Ross's Geese (*Chen rossi*) at 34.5%, in ungulates such as muskox (*Ovibus moschatus*) at 4% and in carnivores such as Arctic fox (*Vulpes lagopus*) at 43.6% (Reiling & Dixon, 2019).

1.10 *Toxoplasma gondii* Structure

Toxoplasma gondii has three major infectious stages: tachyzoites, bradyzoites and sporozoites. During the tachyzoite stage, the parasite replicates rapidly in any cell of an intermediate host, and in the definitive host the replication will occur in the non-intestinal epithelial cells (Dubey et al., 1998). Tachyzoites are crescent shaped with a complex of membranes that envelop the protozoan body except for the most apical region of the cell and the most posterior part of the cell (Dubey, 2023). This parasite's membrane has a distinguishing feature on its most apical region called the conoid. The conoid is crucial to cell infection as when there is an influx of

calcium it can protrude from the membrane allowing it to attach to the host cell (Attias et al., 2020). Within a few days of infection, the tachyzoites slow their metabolism and become bradyzoites. These bradyzoites are encapsulated in a cyst wall and together are known as a tissue cyst (Attias et al., 2020). The bradyzoites will slowly replicate via endodyogeny and the size of the tissue cyst will vary based on where they are in the body and how long they have been replicating (Dubey et al., 1998). These tissue cysts will predominantly be found in the muscular and nervous tissue of the organism but can also be found in other organs as well (Dubey, 1988), (Dubey et al., 1998). Sporozoites are found in sporulated oocysts which are shed by cats and other felids 3-10 days after the original ingestion of the parasite (Attias et al., 2020). They will usually sit in packages of four sporozoites per sporocyst and are enclosed within a single unit membrane (Dubey, 1988).

1.11 *Toxoplasma gondii* Classification

Toxoplasma gondii is a coccidian protozoan parasite and is the only species in the genus *Toxoplasma*. Multilocus polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping of approximately 1,500 samples have yielded 189 different genotypes worldwide (Shwab et al., 2014). In North America, the predominant genotypes are 1,2,3,4 and 5. Genotypes 1 and 3 are the conventional type II lineage, genotype 2 is type III and genotypes 4 and 5 are now classified as the fourth lineage in North America and is known as type 12 (Shwab et al., 2014). In the southern hemisphere there are hundreds of different genotypes that exist whereas in the northern hemisphere there are only a few genotypes that dominate (Shwab et al., 2014). Classification of this parasite will allow for further insight into global transmission pathways and emerging genotypes.

1.12 *Toxoplasma gondii* Transmission

Toxoplasma has a wide range of hosts, but the primary or definitive hosts of the parasite are cats and wild felids, family Felidae, and that is where the sexual reproduction of the parasite occurs (Innes, 2010). The sexual reproduction of this parasite occurs in the felid gut, more specifically the intestines of the felid (Martorelli Di Genova & Knoll, 2020). The sexual life cycle of this parasite begins with the ingestion of the bradyzoites in a prey animal by a felid and then that is where they differentiate into a pre-sexual state where the tissue cyst wall is digested in the stomach and the parasite is released into the small intestine of the cat (Martorelli Di Genova & Knoll, 2020). In the intestine, the formation of merozoites begins and they will either continue to replicate or progress to the final sexual stages of the parasite (Martorelli Di Genova & Knoll, 2020). The two final sexual stages of this parasites life cycle are the microgamete (male) and macrogamete (female), where the macrogametes are intracellular and remain stationary the microgametes can swim around extracellularly using their flagella to fertilize the stationary macrogametes (Martorelli Di Genova & Knoll, 2020). Once fertilized the micro and macrogametes will fuse to create a diploid zygote with a protective wall surrounding it, which can be later shed in the feces as an oocyst to infect other organisms (Martorelli Di Genova & Knoll, 2020). Once released, and favorable conditions arise, the oocyst will sporulate via meiosis to form an oocyst with haploid sporozoites (Martorelli Di Genova & Knoll, 2020).

The asexual reproduction of the parasite follows a different path with the same origin as the sexual path, with the animal ingesting the infective oocysts (Blader et al., 2015). There are two main stages of asexual reproduction: acute and chronic infection (Blader et al., 2015). The acute phase lasts approximately 7-10 days, and this is where the sporozoites inside the oocysts will differentiate into tachyzoites and this is where the parasite will replicate inside the cells until a

maximum of 64-128 parasites have been accumulated, where they will be released to infect neighboring cells (Blader et al., 2015). The second stage of this infection is the chronic cycle, and this is usually characterized by the formation of tissue cysts containing bradyzoites inside the intermediate host cells (Blader et al., 2015). These tissue cysts tend to be localized in the muscle tissue and nervous tissue of the organism that ingests them and can last for the entirety of the organism's life to either lay dormant or activate again once the immune function diminishes (Blader et al., 2015).

Humans can contract the parasite by three main pathways. Following the ingestion of the tissue cysts, they will burst in the stomach and then cause the release of slowly multiplying bradyzoites which will then infect epithelial cells in the small intestine (Blader et al., 2015, Tenter et al., 2000). This can be from either the drinking of unpasteurized milk or the ingestion of meat that has either not been cooked properly or not cooked at all (McConkey et al., 2013). The second main method is through the transmission of infective oocysts in water, soil or fresh produce, known as environmental transmission. The third route of transmission is congenital, whereby the parasite is transferred from a pregnant mother to the fetus. The main risk to the fetus is when the mother contracts *T. gondii* for the first-time during pregnancy (Robert-Gangneux & Dion, 2014) (Robert-Gangneux & Dion, 2014). Of the congenital infections, 60-81% occur during the third trimester of the pregnancy, whereas the risk of transmission in the first trimester is less than 6% (Robert-Gangneux & Dion, 2014). Although the risk of infection may be lower during the first trimester, the severity of the infection caused by *T. gondii* is much greater as it may result in the death of the fetus or severe neurological damage (Montoya & Remington, 2008).

1.13 *Toxoplasma gondii* Diagnostic Methods

With individuals in Canada's North being at increased risk for contracting the parasite it is crucial that diagnostic methods are readily available. This will help lower risks for potential outbreaks in these communities as well as tracking of infected animals.

There are two different types of ELISA that are used in diagnosing *T. gondii* indirect ELISA and sandwich ELISA (Obwaller et al., 1995; Tomasi et al., 1986; Ybañez et al., 2020). In indirect ELISA, the well of the ELISA plate is coated with an antigen and it will bind to the antibody in the sample (Kotresha & Noordin, 2010). Indirect ELISA will most often detect the anti-*Toxoplasma* IgG, IgM and IgA antibodies (Tomasi et al., 1986). There has been improvement as multiple crucial recombinant antigens have been able to be expressed in *Escherichia coli* or yeast (Kotresha & Noordin, 2010). Combinations of these recombinant antigens have proven beneficial by increasing the sensitivity and specificity of the ELISA (Aubert et al., 2000; Kotresha & Noordin, 2010; S. Li et al., 2000; Pfrepper et al., 2005). In sandwich ELISA, the well on the plate is coated with the antigens or antibodies then the sample will be added containing the antigen or antibody causing it to bind to the well. After washing the plate, an enzyme-labeled specific antigen or antibody will be added to the well to bind to the existing complex so that a positive signal can be detected (Dautu et al., 2008). With this ELISA it is possible to detect *T. gondii*-specific IgG, IgM and IgA antibodies as well as circulating antigens (Dautu et al., 2008; B. Lu et al., 2006; Suzuki et al., 2000).

Modified agglutination testing (MAT) is when formalin-fixed tachyzoites are added to a well plate followed by the diluted sera (Desmonts & Remington, 1980). The positive samples will form a thin layer, and negative samples will form a pellet at the bottom of the well, these will be observed under a microscope. This test is used to detect IgG but has issues when it comes to

sensitivity and selectivity as it has the possibility of non-specifically binding to the IgM on the surface of the parasite (MacRì et al., 2009; C. H. Zhu et al., 2012).

1.14 *Toxoplasma gondii* Detection Methods

Toxoplasma gondii is a parasite that can be transmitted to humans via the consumption of contaminated food or water. Most often if the meat is cooked to the appropriate temperature based on Health Canada guidelines (Health Canada, 2020) it will be safe for human consumption. The individuals at risk are those who will be consuming country food, as most of the preparations are uncooked. Therefore, it is crucial that detection methods are in place to test food and water samples for potential contamination with the parasite. This will allow for repeatable and accurate testing of both food and water samples to help minimize potential public health crises. Currently there are two key methods of detection for *Toxoplasma gondii* in foods, both of which have both advantages and disadvantages. These key detection methods are PCR and bioassay.

Polymerase chain reaction (PCR) is a crucial tool to aid in the detection of *T. gondii* infection. It is used most often in conjunction with serology as it requires a small amount of DNA and it allows for rapid and selective amplification of genes to identify the organism in blood, serum or tissue samples (Opsteegh et al., 2010; Su et al., 2010). The original method used to detect *T. gondii* was targeting the B1 gene in 1989 by Burg et al. (Burg et al., 1989) and was widely used to diagnose infection in immunocompromised patients (Ho-Yen et al., 1992; Khalifa et al., 1994; Lamoril et al., 1996; Parmley et al., 1992). There was a method discovered using the 529 bp repeat element by Homan et al. in 2000 (Homan et al., 2000). This study concluded that the sensitivity of using this gene in comparison to the B1 gene was 10-100 times greater (Homan et al., 2000). *T. gondii* is a difficult parasite to detect as most often times the tissue cysts are located randomly throughout the tissues and if the tissue cyst is not selected during sampling, then the sample could

provide a false negative result. This issue was attempted to be solved by Opsteegh et al. in 2010 using a method that allowed for more tissue to be screened in the PCR (Opsteegh et al., 2010). This method called Magnetic capture PCR uses magnetic beads that will attach to labeled *T. gondii* DNA to allow for more selectivity when a large sample is tested (Opsteegh et al., 2010). This method has allowed for larger samples to be screened for *T. gondii*, meaning that there is an increased chance of detecting *T. gondii* DNA given that tissue cysts are heterogeneously distributed throughout the tissue (Opsteegh et al., 2010).

Bioassays are used to confirm the presence of viable *T. gondii* in host tissues through the experimental infection of mice or cats. Tissues such as muscle tissue, brain tissue, lymph nodes are taken from the experimentally infected animals to isolate the parasite (Dubey et al., 2004, 2013). There are some downsides to this method of detection as it is resource intensive to purchase and take care of the animals for the approximately 6 weeks required to have a viable infection. Therefore, it is difficult to use for large scale screening of country foods. Bioassays also pose ethical issues due to animal use.

1.15 Rationale

Zoonotic pathogens present a serious public health risk for communities in Canada's North. The incidence of infection is increased likely due to the consumption of contaminated country food and water. Country foods are culturally significant and extremely nutrient dense. They, however, increase the risk of contracting zoonotic pathogens as the traditional preparation methods of country foods are uncooked (raw, dried, smoked, etc.). Although there has not yet been an established connection, climate change is most likely increasing the risk of contracting a zoonotic pathogen. As a result of climate change the migration patterns of the arctic animals will change. Thus, extending the northward migration of these arctic animals that are traditional sources of

country food, leading to an increase in the exposure to zoonotic pathogens (Reiling & Dixon, 2019).

1.16 Hypothesis and Objectives

Climate change will increase the prevalence of zoonotic pathogens in Canada's North thereby increasing the associated risk of human infection.

The primary aim of this thesis is to identify the current prevalence of Hepatitis E virus and *Toxoplasma gondii* in keystone country food species such as caribou, ringed seal and beluga. These three animal groups were selected to begin this study as they had the largest variety of samples when selecting, meaning that they had the widest variety of tissues that were obtainable. The second reason that these animals were selected was because they are among the most common country foods in the traditional Northern Indigenous diet (Caughey et al., 2021). Although *T. gondii* has been reported in several studies on Arctic wildlife, HEV has never been characterized in marine mammals in previous literature.

The secondary aim is to optimize the methods to test and confirm the presence of these pathogens for future testing on country food samples. It is crucial that any of the results obtained are communicated back to the communities or scientists that were gracious enough to give these samples for testing. This will allow for a greater understanding of what pathogens now potentially infect these keystone country food species, and to assist in mitigation measures.

2. Materials and Methods

2.1 Northern Wildlife Samples

These samples were shared by the scientists from: Northern Ontario (ON) from Dr. Devin Empry, Northern Manitoba (MB), Nunavut (NU), Northwest Territories (NT) from Dr. Ole Nielsen, northern Quebec (Nunavik Region) from Dr. Geraldine Gouin and Yukon (YT) from Dr. Jane Harms.

These were convenience samples provided by local hunters and biologists. This meant that the samples received were whatever tissues and blood that was offered by the local communities. All these samples were from keystone country food species and were the traditional diet of the communities that shared these samples. These samples were taken by the local hunters in locations that are traditionally hunted. It was crucial not to take more samples than what was given out of convenience as the animals were crucial to the nutrition and culture of these Northern Indigenous communities. The samples were representative of the immediate region of which they were harvested, but not representative of a larger region in Canada's North.

2.1.1 Serum and Whole Blood Samples

Both whole blood and serum samples were collected by hunters and biologists. Samples received were from keystone wildlife species including caribou, ringed seal and beluga as they are most often eaten uncooked by the northern indigenous population.

Caribou samples were collected from herds near Gods Lake, MB in 2011, Pen Island, NU from 2011-2013, Cape Churchill, MB in 2012 and Berens-Sydney Range, ON from 2019-2024.

Ringed seal samples were sampled from Arviat, NU in 2015, Coral Harbour, NU in 2016, Pangnirtung, NU in 2017, Pond Inlet, NU in 2017 and Sanikiluaq, NU from 2011-2017.

Beluga samples were collected from East Whitefish, NT in 2017, Hendrickson Island, NT; Kendall Island, NT in 2017 and Tuktoyaktuk, NT in 2017 (Table 1a, 1b and 1c).

The samples were shipped to the Banting Research Centre (Health Canada) in Ottawa from their respective regions in large coolers filled with freezer packs to maintain a freezing temperature. Each shipment was opened upon arrival and inspected to see if any of the samples were compromised in any way or thawed.

Blood and serum samples were stored at -80 °C until nucleic acid was extracted. The approximate volume of each serum sample was 1.5 mL and each blood sample was 2 mL.

Table 1a. Number of Caribou Samples Tested by Location and Sample Type

Sample Type	RNA	Serum	Blood	Brain	Heart	Liver	Muscle	Grand Total
Berens-Sydney Range, ON	-	36	-	-	-	-	-	36
Cape Churchill, MB	-	11	-	-	-	-	-	11
Gods Lake, MB	-	-	4		-	-	-	4
Northern Ontario	-	130	-	-	-	-	-	130
Nunavik, QC	-	-	20	5	1	15	1	42
Pen Island, NU	-	12	18	-	-	-	-	30
UNK	-	3	-	-	-	-	-	3
Quebec	20	-	-	-	-	-	-	20
Total	**20**	**192**	**42**	**5**	**1**	**15**	**1**	**276**

Note: The RNA samples were isolated prior to receiving the samples. Brain, heart, liver and muscle samples were sub sectioned from the entire organ prior to receiving the sample.

Table 1b. Number of Ringed Seal Samples Tested by Location and Sample Type

Sample Type	Blood	Brain	Diaphragm	Heart	Liver	Muscle	Grand Total
Arviat, NU	1	-	-	-	-	-	1
Coral Harbour, NU	14	-	-	-	-	-	14
Nunavik, QC	-	8	2	1	6	7	24
Pangnirtung, NT	1	-	-	-	-	-	1
Pond Inlet, NU	20	-	-	-	-	-	20
Sanikiluaq, NU	136	-	-	-	2	-	138
Total	**172**	**8**	**2**	**1**	**8**	**6**	**198**

Note: Brain, diaphragm, heart, liver and muscle samples were sub sectioned from the entire organ or muscle prior to receiving the sample.

Table 1c. Number of Beluga Samples Tested by Location and Sample Type

Sample Type	Blood	Liver	Grand Total
East Whitefish, NT	10	-	10
Hendrickson Island, NT	53	8	61
Kendall Island, NT	8	1	9
Tuktoyaktuk, NT	1	-	1
Total	**72**	**9**	**81**

Note: Liver samples were sub sectioned from the entire organ prior to receiving the sample.

2.1.2 Tissue Samples

Tissue samples were collected by local hunters and scientists in various commonly hunted regions of Canada's North listed above in section 2.1. The tissue samples that were provided included liver, muscle tissue, heart, brain and diaphragm (Table 1a, 1b and 1c). These were selected as they are the predilection sites of these pathogens.

The tissue samples were shipped to the Banting Research Centre from their respective regions in large coolers filled with freezer packs to maintain a freezing temperature. Each shipment was opened upon arrival and inspected to see if any of the samples were compromised or thawed.

The tissue samples were thawed and subsampled using sterile scalpel blades, that were changed in between each sample, into smaller aliquots of 10 grams for *Toxoplasma gondii* testing (Magnetic capture method) and 2 grams for hepatitis E testing (RNA Extraction Method from Liver). These subsamples were taken from the interior of the tissue samples as an extra precaution against cross contamination.

These samples were stored at -80 °C until nucleic acids were extracted from the samples. The tissues ranged in size from as small as 1 g of tissue all the way up to 200 g of tissue.

2.2 RNA Extraction for Hepatitis E Virus

HEV RNA was extracted from the caribou serum samples using the QIAamp® Viral RNA Mini kit (Qiagen, Venlo, LI). This kit uses carrier RNA to enhance the binding of the viral nucleic acids to the membrane in the sterile spin column. The carrier RNA is also important as it is added at a much higher concentration than the viral RNA, therefore reduces the chance of RNAses degrading the viral RNA. This protocol was followed, and the extracted RNA was stored in the -80 °C until further testing.

2.3 Hepatitis E Virus ELISA

Serum samples from the keystone species were thawed and then MP Diagnostics HEV ELISA 4.0v kit (MP Biomedicals, Santa Ana, CA) was used to detect anti-HEV antibodies in the serum. The test was done following the instructions provided with the MP Diagnostics HEV ELISA 4.0v. The ELISA kit uses a proprietary recombinant antigen, that is highly conserved between different HEV strains. This is used to detect IgG, IgM and IgA against HEV.

2.4 *Toxoplasma gondii* ELISA

The analysis of the serum samples was done using an ELISA kit. The ID screen® Toxoplasmosis Indirect Multi-species ELISA kit (Innovative Diagnostics, Saint-Eustache, QC) was selected due to its reactivity with different host species making it ideal for this project. The samples were tested according to the instructions provided to ensure the best results based on the sample type selected to test, either blood or serum. For serum samples, a 1:10 dilution was made with the dilution buffer prior to incubating the samples in the microplate. For any meat juice samples or whole blood, a 1:2 dilution was made prior to incubation in the microplate.

2.4.1 Cross Reactivity Testing

There were some concerns with this kit cross reacting with *Sarcocystis* spp., *Neospora caninum*, *Besnoitia besnoiti*, and *Hammondia hammondi* as they are similar genetically and taxonomically to *T. gondii*. Cross reactivity was ruled out with these parasites through experiments done by Innovative Diagnostics as they tested 15 bovine serum samples that were positive for *Neospora caninum* using the ID screen® Toxoplasmosis Indirect Multi-species ELISA kit and found that these bovine serum samples were not positive for *T. gondii*. Innovative Diagnostics also tested 30 bovine serum samples that were positive for *Besnoitia besnoiti* on the ID screen®

Toxoplasmosis Indirect Multi-species ELISA kit and found that these samples were not reporting false positives. Bouchard et al. (Bouchard et al., 2022) tested cross reactivity of the ID screen® Toxoplasmosis Indirect Multi-species ELISA kit using feline serum that was positive for *Hammondia hammondi* provided to them from the Centre for Food-borne and Animal Parasitology (CFAP) in Saskatoon, SK. They found that the ELISA sensitivity was 94% for *T. gondii*. Finally, cross reactivity testing was done for *Sarcocystis* spp. by taking four *Sarcocystis* spp. positive samples, including three harp seal samples (two diaphragms and one heart) and one ringed seal muscle sample. These samples were tested on the ID screen® Toxoplasmosis Indirect Multi-species ELISA kit where it was found that these four *Sarcocystis* spp. positive samples did not cross react. The ID screen® Toxoplasmosis Indirect Multi-species ELISA kit was selected as it was using a p30 (SAG1) antigen along with an anti-multispecies conjugate allowing for broad reactivity among wildlife species.

2.5 EASYMAG DNA and RNA Extraction

Whole blood samples were thawed and in order to continue with testing it was required that the nucleic acids were isolated. The EasyMag uses silica beads (Biomerieux, Saint-Laurent,QC) to capture the desired nucleic acid which is then attracted by the nuclisens® easymag® magnet allowing for the nucleic acids to be purified by wash steps. The mixture is heated to 70°C for 10 min and the nucleic acid is released into the elution buffer where it was stored in the -80 °C freezer for further testing.

2.6 *Toxoplasma gondii* Magnetic Capture qPCR

Magnetic Capture qPCR was selected for this project as it allows for larger samples to be screened for *T. gondii*, meaning that there is an increased chance of detecting *T. gondii* DNA given that tissue cysts are heterogeneously distributed throughout the tissue (Opsteegh et al., 2010).

Tissue samples were weighed in sterile weigh boats after being thawed and cut into small 1 cm by 1 cm cubes to ensure even digestion. These cubes were weighed and stored sterile Falcon™ 50 mL High Clarity Conical Centrifuge Tubes (Fisher, Waltham, MA) with approximately 10 g of tissue per tube. The magnetic capture qPCR method was originally developed by Opsteegh et al. (Opsteegh et al., 2010) and the procedure from that study was followed. The cut tissue was then put into a stomacher bag with 25 mL of cell lysis buffer containing 100 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl, 40 mg/l proteinase K. This sample was then homogenized on high for 2 min and then incubated in a 55 °C water bath overnight.

After the incubation the sample was homogenized for one additional min on high. 25 mL of homogenate was then transferred into a centrifuge tube and centrifuged at 3500 x g for 45 min. Once the centrifugation was completed, 12 mL of supernatant was transferred to a fresh centrifuge tube to continue.

The next step of the procedure was to remove the free biotin from the sample. This was done by heating the liquid in a heating block for 10 min at 100 °C. While the sample was being heated, 50 µL of streptavidin sepharose (Cytiva, Marlborough, MA) per sample was washed three times in sterile phosphate buffered saline (PBS). Once the samples have been cooled the 50 µL of streptavidin sepharose was added to the sample. This sample was then left to incubate at room temperature for 45 min rotating at 10 RPM to allow for streptavidin-biotin binding. Once completed the sample was centrifuged at 3500 x g for 15 min and 10 mL of the biotin free supernatant was transferred to a sterile Falcon™ 50 mL High Clarity Conical Centrifuge Tubes (Fisher, Waltham, MA).

Sequence specific primers were added to the mixture in order to make this magnetic capture sequence specific to *T. gondii*. Therefore, 2 µl of primer Tox-CapF and Tox-CapR were added to each supernatant (Table 2). The supernatants were then heated to 95 °C for 15 min to denature all DNA. These samples were then transferred to a shaking water bath that was set for 55 °C and left to allow for hybridization between capture oligonucleotides and *T. gondii* DNA for 45 min. These samples needed to be cooled to room temperature once again rotating at 10 rpm for 15 min. While cooling the samples, 80 µl of M-270 Streptavidin Dyna beads were washed, per sample tube (Invitrogen, Waltham, MA). The M-270 Streptavidin Dyna beads were washed in sterile 1 x Binding & Washing (B&W) buffer (5 mM Tris HCl pH 7.5, 0.5 mM EDTA pH 8.0, 1 M NaCl) according to the manufacturer's instructions. Once the sample was cooled, the washed M-270 Streptavidin Dyna beads were added and 2 mL of 5 M NaCl was added to each supernatant sample and the samples were incubated rotating (10 rpm) at room temperature for 60 min. The complex of streptavidin bead and biotin-labelled capture-oligonucleotide with hybridized *T. gondii* DNA was isolated by using the DynalMPC-1magnet (Invitrogen, Waltham, MA). The tube was placed in the magnet for 10 min and the supernatant was removed with a disposable Pasteur pipette.

Finally, the beads were washed twice in sterile 1 x B&W buffer and resuspended in 50 µl of distilled water in a sterile 1.5 mL Eppendorf™ Safe-Lock Tubes (Eppendorf, Hamburg, HH). After washing the bead suspension was heated at 100 °C for 10 min to release *T. gondii* DNA from the beads. The beads were placed in the Dynal MPC-S magnet (Invitrogen, Waltham, MA), and the supernatant was immediately transferred to a clean 1.5-ml tube. The beads were then discarded.

The DNA was stored in a -20 °C freezer for quantitative PCR testing. The qPCR parameters follow the methods outlined in the Opsteegh et al. study. The primer set used was targeting the 529-bp repeat element.

2.6.1 Artificially Contaminated Chicken

Toxoplasma gondii oocysts were diluted in series from 10^6 to 10^2 in 1 mL of water and then placed in a sterile Falcon™ 50 mL High Clarity Conical Centrifuge Tubes (Fisher, Waltham, MA). Approximately 10 g of cut chicken breast purchased from a retail grocery store was added to each tube in a separate sterile hood. These samples were mixed via inversion to cover the sample completely. These were then frozen to simulate the freeze thaw cycle of a country food sample. These were then put through the magnetic capture method and qPCR to detect *T. gondii* DNA. This was done to evaluate the method in the lab and to get an idea as to how sensitive magnetic capture is for *T. gondii* DNA.

2.6.2 Artificially Contaminated Northern Wildlife

Tissue samples from belugas that were previously determined to be negative for *T. gondii* were spiked with *T. gondii* oocysts and retested using the magnetic capture qPCR method. In order to replicate the natural infection, the closest for control purposes, *T. gondii* oocysts were diluted in series from 10^6 to 10^2 in 1 mL of water. In a different sterile hood, the oocysts were placed on approximately 10 g of cut country food samples to simulate infection.

2.6.3 Naturally Contaminated Northern Wildlife

Tissue samples from belugas that were previously confirmed positive for *T. gondii* were retested using the magnetic capture method in order to confirm positivity and ensure that the method was working properly. These samples were then run on qPCR to quantify the DNA.

Table 2. List of primers for *T. gondii* Magnetic Capture PCR

Name	Sequence 5′-3′	Ref
Tox-CapF	CTTGGAGCCA CAGAAGGGAC AGAAGTCGAA GGGGACTACA GACGCGATGC CGCTCCTCCA GCCGTCTTGG	(Opsteegh et al., 2010)
Tox-CapR	AAGCCTCCGA CTCTGTCTCC CTCGCCCTCT TCTCCACTCT TCAATTCTCT CCGCCATCAC CACGAGGAAA	(Opsteegh et al., 2010)

2.7 Hepatitis E Virus Droplet Digital PCR

Previously extracted HEV RNA was removed from the freezer and placed on ice until added to the mastermix. Each sample was prepared following the method outlined in a paper by Cecillon and Nasheri (Cecillon & Nasheri, 2024). The One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, Hercules, CA) included One-Step RT-ddPCR Supermix, Reverse transcriptase (Bio-Rad) and 300 mM Dithiothreitol (DTT) (Bio-Rad) to prepare the master mix. The mastermix was made in sterile 1.5 mL Eppendorf™ Safe-Lock Tubes (Eppendorf, Hamburg, HH) by mixing 6 µL of Supermix, 1 µL of DTT, 1 µL of 18 µM forward primer (Table 3), 1 µL of 18 µM reverse primer (Table 3), 1 µL of 5 µM HEV FAM probe (Table 3), 5.5 µL of ultrapure water and 2.5 µL of reverse transcriptase. 18 µL was added to each reaction vessel to which 5 µL of RNA was added in a separate sterile hood to complete the preparation for the droplet generation step.

The samples were transferred to DG8™ Cartridges for QX200™/QX100™ Droplet Generator and gaskets (Bio-Rad) along with 70 µL of droplet generation oil into the bottom wells of the DG8 cartridge. The full DG8™ Cartridges were placed in the QX200 Droplet Generator (Bio-Rad). The plate was then sealed and placed in the C1000 Touch™ thermal cycler (Bio-Rad) following the cycling parameters in Cecillon and Nasheri (Cecillon & Nasheri, 2024). These parameters were: 50°C for 60 min, then 95°C for 10 min, 95°C for 30 seconds with a ramp speed of 2°C/s, 55°C for NoV GI, NoV GII and HEV for 1 min with a ramp speed of 2°C/s. Loop back to the previous two steps for 40 cycles. After the 40 cycles proceed with 98°C for 10 min and finally hold at 12°C.

The results were read and analyzed using QX200 Droplet Reader (Bio-Rad) and the corresponding QX Manager software.

Table 3. List of primers and probe for HEV ddPCR

Name	Sequence 5′-3′	Reference
HEV Forward	GGT GGT TTC TGG GGT GAC	(Jothikumar et al., 2006)
HEV Reverse	AGG GGT TGG TTG GAT GAA	(Jothikumar et al., 2006)
HEV FAM	TG ATT CTC A/ZEN/G CCC TTC GC/3IABkFQ	(Jothikumar et al., 2006)

ZEN and 3IABkFQ are quenchers. Quenchers are essential as they bind with fluorophore causing the fluorescence of the fluorophore to decrease through fluorescence resonance energy transfer (FRET) until cleavage (Nazarenko et al., 2002).

2.8 RNA Extraction Method from Liver

HEV RNA was extracted from liver samples using Dr. Reimar Johne's method of isolation (Trojnar et al., 2020). This method was selected as it is currently in the process of being approved as the ISO standardized method for "Isolation of viral RNA from liver and detection of Hepatitis E virus using virus-specific real time RT-PCR" (Trojnar et al., 2020). This method involved the homogenization of 2 g of liver samples using ceramic beads in sterile Phosphate-buffered saline (PBS), then Precellys® 2 mL Sample Grinding Garnet Flakes Kit (GK60) (Bertin Technologies, Montigny-le-Bretonneux, YV) and finally the resulting homogenate is run through the QIAgen RNeasy mini kit to isolate the viral RNA. This method is verified using a control virus to assess the recovery rate of the procedure. This process was done to ensure that there is adequate recovery of RNA, and that the RNA was of good quality. Recovery rate was calculated by dividing the concentration of the process control virus of the sample recovered by the concentration of the control virus that was added to the sample prior to the extraction.

2.9 Hepatitis E Virus Real Time-PCR (RT-PCR)

2.9.1 Hepatitis E Virus Hemi-nested ORF1

The first trial made to sequence the RNA involved the use of the primers and cycling parameters of Drexler et al. (2012) (Drexler et al., 2012). This protocol was targeting a 337 bp viral RNA-dependent RNA polymerase (RdRp) in ORF1 between positions 4228-4565 (Figure 1). The primer set was hemi-nested meaning that there are two rounds of PCR. The first round was conventional PCR with a forward and reverse primer and the second round using a reverse primer that was inside that of the first round allowing for higher selectivity for amplification. This protocol was selected due to its broad reactivity with novel HEV strains.

2.9.2 Hepatitis E Virus ORF2

The second trial made to sequence the RNA involved the use of the primers and cycling parameters of Baylis et al. (2011) (Baylis et al., 2011). This protocol was targeting a 347 bp sequence in the ORF2 region of the genome between positions 5972-6319 (Figure 1). This was selected as it was described as a good sequencing protocol to genotype the virus.

2.9.3 HEVnet ORF2

The third trial made to sequence the RNA involved the use of the nested primers and cycling parameters of Boxman et al. (2017) (Boxman et al., 2017). This protocol was targeting a 493 bp sequence in the ORF 2 region of the genome between 5961-6455 (Figure 1). This was a completely nested primer set as the second round of PCR was using primers that were inside the first-round product, to increase selectivity of amplification. This was selected as this was recognized by HEVnet (National Institute for Public Health and the Environment, 2019) as the most widely accepted method for PCR detection and sequencing of HEV.

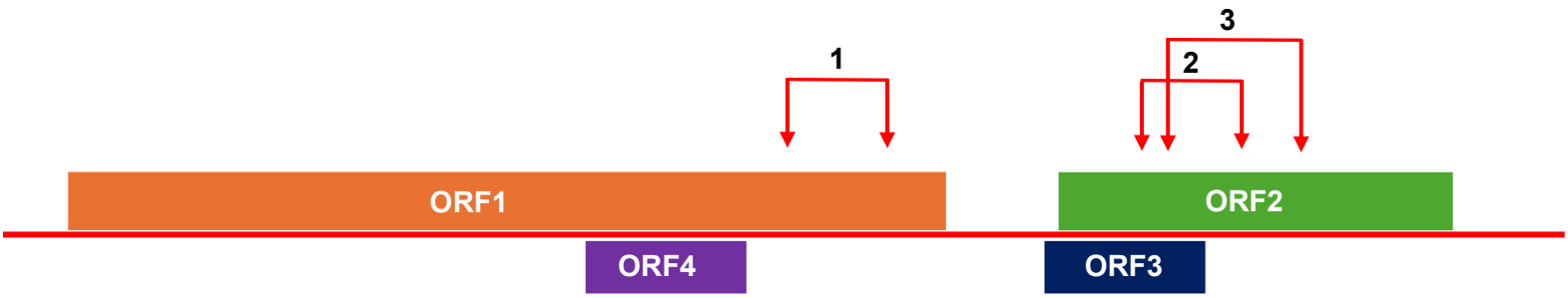


Figure 1. HEV Genome with Binding Sites for HEV RT-PCR Primers. The first site corresponds to the Drexler et al. (2012) primer set. The second site corresponds to the Boxman et al. (2017) primer set. The third corresponds to the Baylis et al. (2011) primer set.

2.10 Sequencing

HEV RNA from presumptive positive samples was stored separately for sequencing to confirm the positivity of the samples. Samples were cycled at the parameters outlined based on the primer sets (Table 4). HEV Hemi-nested ORF1 was done according to Drexler et al. (2012) (Drexler et al., 2012), HEV ORF2 using Baylis et al. (2011) (Baylis et al., 2011) and HEVnet ORF2 using Boxman et al. (2017) (Boxman et al., 2017)

After imaging, the desired bands were removed using a sterile scalpel and the RNA was extracted using the Qiagen QIAquick Gel Extraction Kit. After the products were extracted, the purified PCR product was labeled using the "Big DyeTerminator" kit (v3.1, Applied Biosystems, Waltham, Massachusetts). For each sample, two separate reactions were done, one with the forward primer (5uM) and another with the reverse primer (5uM). The PCR products were then cycled according to the BDT sequencing instructions which are, 96 °C for 1 min, then 25 cycles of: 96°C for 10 seconds, 50°C for 5 seconds with a ramp speed of 1°C/s, 60°C for 4 min with a ramp speed of 1°C/s, then repeat back to 96°C for 10 seconds with a ramp speed of 1°C/s. These samples were run at 170 V for 50 min on 2% agarose gels using a 50 bp ladder (Invitrogen, Waltham, Massachusetts).

These samples were cleaned up using the Promega Wizard ® Magnesil ® Sequencing Reaction Clean-up System. The cleaned-up product was then placed in the Applied Biosystems 3500 Genetic Analyzer and the results were analyzed using SeqScape3 and BioEdit programs. Sequences that had good quality were then blasted against the NCBI databases to see if they matched the reference sequences.

Table 4. Primers used for HEV RT-PCR and sequencing

Name	Sequence 5′-3′	Reference
	HEV Hemi-nested ORF1	
Forward	ACYTTYTGTGCYYTITTTGGTCCITGGTT	(Drexler et al., 2012)
Reverse Out	GCCATGTTCCAGAYGGTGTTCCA	(Drexler et al., 2012)
Reverse In	CCGGGTTCRCCIGAGTGTTTCTTCCA	(Drexler et al., 2012)
	HEV ORF2	
Forward	GTYATGYTYTGCATACATGGCT	(Baylis et al., 2011)
Reverse	AGCCGACGAAATYAATTCTGTC	(Baylis et al., 2011)
	HEVnet ORF2	
Forward Out	AAYCARGGITGGCGYTCIGTIGARAC	(Boxman et al., 2017)
Forward In	GAGGAGGAAGCTACCTCYGGYYTIGTIATGCTYTGYAT	(Boxman et al., 2017)
Reverse Out	GARAAIGGRCGIGAIGGRGCIGG	(Boxman et al., 2017)
Reverse In	GGAGAAGGAGTTGGTCGRTCYTGYTCRTGYTGRTT	(Boxman et al., 2017)

Mixed bases in degenerate primers and probes are as follows: Y, C or T; R, A or G; I.

3. Results

3.1 Method validation

Throughout the various experiments there were different types of validations that needed to be done in order to demonstrate that the method was functioning properly with accurate results.

3.1.1 Hepatitis E Virus Liver Extraction Efficiency

To test the liver extraction method a controlled amount of Murine Norovirus (MNV) was added to the 32 livers after the first homogenization step in order to determine the extraction efficiency of the protocol. The extraction efficiency was calculated by the ratio of the obtained genome copies of Murine Norovirus (MNV) after the liver extraction using ddPCR to the number of Murine Norovirus (MNV) genome copies added to the original liver sample prior to the extraction method and multiplied by 100. Any extraction efficiency calculation yielding one percent or higher was considered successful. If the extraction efficiency was below one percent the experiment was redone. The stock was diluted to 10^3 genome copies of Murine Norovirus (MNV) per 10 µL and ddPCR was conducted on the samples yielding an approximate mean extraction efficiency of 23.9% (Table 5).

Table 5. Murine Norovirus (MNV) extraction efficiency using the Reimar liver extraction method. Testing the extraction efficiency of the Reimar liver extraction method using murine norovirus (MNV) as a control. Liver samples were spiked with 10^3 genome copies per 10 µL. ddPCR was run to determine the amount of MNV in each sample.

Sample	Genome copies in total RNA	Extraction Efficiency (%)
10^4 Stock MNV	1242.60	N/A
BMH-GRDI-226	22.18	17.85
BMH-GRDI-227	57.50	46.27
BMH-GRDI-228	5.74	4.62
BMH-GRDI-229	29.47	23.72
BMH-GRDI-230	54.73	44.04
BMH-GRDI-231	26.72	21.51
BMH-GRDI-232	32.37	26.05
BMH-GRDI-233	5.16	4.15
BMH-GRDI-234	33.59	27.03
BMH-GRDI-235	9.48	7.63
BMH-GRDI-236	54.13	43.56
BMH-GRDI-237	53.74	43.25
BMH-GRDI-238	15.21	12.24
BMH-GRDI-239	5.89	4.74
BMH-GRDI-240	46.79	37.66
BMH-GRDI-518	44.46	35.78
BMH-GRDI-519	12.23	9.85
BMH-GRDI-522	31.14	25.06
BMH-GRDI-527	45.78	36.85
BMH-GRDI-532	24.10	19.40
BMH-GRDI-535	59.59	47.96
BMH-GRDI-539	9.94	8.00
BMH-GRDI-540	5.78	4.65
BMH-GRDI-541	16.01	12.88
BMH-GRDI-1349	45.64	36.73
BMH-GRDI-1350	43.55	35.05
BMH-GRDI-1351	6.44	5.18
BMH-GRDI-1352	20.10	16.17
BMH-GRDI-1353	23.65	19.03
BMH-GRDI-1354	27.26	21.94
BMH-GRDI-1355	31.08	25.01
BMH-GRDI-1356	49.89	40.15

3.1.2 Magnetic Capture qPCR

To validate the Magnetic capture qPCR method, the test was run with samples that had been confirmed positive via conventional PCR in a previous project. These included two beluga brains and one beluga heart from the St. Lawrence Estuary, QC. These were selected as candidates for this validation as they were confirmed and genotyped in a study by Iqbal et al. in 2018 (Iqbal et al., 2018). These samples showed similar levels to the previous positive controls of spiked chicken and were used as another set of controls for future experiments.

Table 6. Comparison of average Cq values of the different positive controls. Comparison of positive controls that were used in magnetic capture qPCR. Samples were tested in triplicate. Average Cq values were calculated.

Samples tested (no. oocysts)	Average Cq
Oocyst suspension (10^5)	31.62
chicken breasts (10^5)	36.49
chicken breasts (10^4)	36.42
St. Lawrence Estuary beluga heart	25.01
St. Lawrence Estuary beluga brain	26.04

3.2 Hepatitis E Virus ELISA

3.2.1 Serum

This ELISA test was done to see if the caribou serum contained IgG, IgM and IgA against HEV. These caribou serum samples were tested using ELISA kit MP Diagnostics HEV ELISA 4.0v. There was a total of 148 caribou serum samples tested, 48 samples from Manitoba from 3 different locations: Gods Lake, Cape Churchill and an Unknown location. There were 30 samples from Pen Island, Nunavut and the remaining 100 serum samples were from Northern Ontario. To be considered positive the sample needed to have an absorbance value greater than 0.20 plus the average absorbance of the negative reactive controls. Out of the tested samples, there were four positives from Northern Ontario and none from Manitoba. Therefore, the overall seroprevalence in the caribou samples was 2.7 % (Table 7). The OD values for these four samples are 0.3725 for sample BMH-GRDI-080, 0.2725 for sample BMH-GRDI-098, 0.2475 for sample BMH-GRDI-108 and 0.376 for sample BMH-GRDI-109.

Table 7. HEV ELISA testing results of caribou serum. Test to detect IgG, IgM and IgA against HEV in the serum. Serum samples were tested in triplicate. UNK = unknown location

Location	Samples Tested	ELISA positive	Prevalence (%)
Northern Ontario	100	4	4
Gods Lake, MB	4	0	0
Cape Churchill, MB	11	0	0
Manitoba (UNK)	3	0	0
Pen Island, NU	30	0	0
Total	148	4	2.7

3.2.2 Other Samples

Testing was originally done on caribou liver juice samples, the liquid that was in the container along with the caribou liver, as well as the caribou serum samples. As HEV localizes in the liver tissue so testing was done to see the presence of any IgG, IgM and IgA against HEV. All the caribou liver samples tested produced an absorbance result that was positive. Through further consultation with the manufacturer, it was determined that this test was not validated for anything other than serum, and these results were not counted towards potential positive samples. Other methods were used to test these caribou liver samples.

3.3 *Toxoplasma gondii* ELISA

3.3.1 Cross Reactivity Analysis

Earlier studies have been done to assess whether the ID screen® Toxoplasmosis Indirect Multi-species ELISA kit (Innovative Diagnostics) would react with other closely related coccidian parasites. The kit was previously tested for *Neospora caninum, Besnoitia besnoiti,* and *Hammondia hammondi* by both the manufacturer or by Bouchard et al. (Bouchard et al., 2022). The conclusion of those studies was that they do not cross react with this ELISA kit. As the cross reactivity of *Sarcocystis* spp. was unknown, this was then determined. Four confirmed *Sarcocystis* positive samples were used from a previous study and the juices from around the sample were collected and used in the assay. These samples included three harp seal samples (two diaphragms and one heart) and one ringed seal muscle sample. To be considered positive for ID screen® Toxoplasmosis Indirect Multi-species ELISA kit the sample to positive ratio (S/P%) was calculated according to $S/P\,\% = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100$, where OD is the optical density, PC is positive control and NC is negative control. If the S/P% ≥ 50% then the sample is positive

according to the manufacturer. The four samples were not positive with the *Toxoplasma* ELISA kit as the S/P% for these samples were 27.7, 7.3, 17.1 and 4.5, meaning that *Sarcocystis* spp. did not cross react.

3.3.2 Serum from Caribou

Testing was done on the serum samples from caribou, a keystone country food species using the ID screen® Toxoplasmosis Indirect Multi-species ELISA kit. These tests were targeting the p30 (SAG1) antigen along with an anti-multispecies conjugate allowing for broad reactivity with multiple different species. There were 50 caribou serum samples tested from Northern Ontario, 3 caribou serum samples from Gods Lake, MB, 11 caribou serum from Cape Churchill and 29 caribou serum samples from Pen Island, NU. Out of the 93 caribou serum samples, two were positive on the ELISA test, one from northern Ontario and one from Cape Churchill, MB. The S/P% of the caribou samples were 65 from Northern Ontario and 137 from Cape Churchill, MB, indicating that these samples were positive. The seroprevalence in the caribou samples was 2.2 % (Table 8).

Table 8. *Toxoplasma gondii* ELISA testing results of caribou serum. Serum samples were tested in triplicate.

Location	Samples Tested	ELISA positive	Prevalence (%)
Northern Ontario	50	1	2
Gods Lake, MB	3	0	0
Cape Churchill, MB	11	1	9
Pen Island, NU	29	0	0
Total	93	2	2.2

3.3.3 Whole Blood from Ringed Seals and Beluga

The ID screen® Toxoplasmosis Indirect Multi-species ELISA kit was rated to test both serum and whole blood. The serum was a 1:10 dilution whereas for the blood it was a 1:2 dilution to test for the antigen in the whole blood samples. Blood samples were assessed using the same S/P% guidelines as the previous experiments. Out of these samples there are 4/14 (28.6 %) ringed seal samples from Coral Harbour, NU, 2/21 (9.5%) ringed seal samples from Pond Inlet, NU and 6/102 (5.9%) ringed seal samples from Sanikiluaq that were positive using the ID screen® Toxoplasmosis Indirect Multi-species ELISA kit (Innovative Diagnostics). Therefore, the positive prevalence from the ringed seal whole blood samples was 8.6 % (Table 9). There were no positive beluga blood samples detected using the ID screen® Toxoplasmosis Indirect Multi-species ELISA kit.

Table 9. *Toxoplasma gondii* ELISA testing results of ringed seal and beluga whole blood. Serum samples were tested in triplicate.

Location	Samples Tested	ELISA positive	Prevalence (%)
	Ringed Seal		
Arviat, NU	1	0	0
Pangnirtung, NT	1	0	0
Coral Harbour, NU	14	4	28.6
Pond Inlet, NU	21	2	9.5
Sanikiluaq, NU	102	6	5.9
Total	139	12	8.6
	Beluga		
East Whitefish, NT	10	0	0
Hendrickson Island, NT	33	0	0
Kendall Island, NT	8	0	0
Tuktoyaktuk, NT	1	0	0
Total	52	0	0

3.4 Hepatitis E Virus ddPCR

Droplet digital PCR (ddPCR) was used as a screening test for HEV on serum and liver samples from a variety of wildlife, with positive samples then undergoing further testing to try to determine the genotype of HEV. There was a total of 483 samples tested and there were 25 positives. Caribou accounted for 10 of the positives, 8 of them from the serum and 2 from liver samples. The 8 serum samples were from Northern Ontario and the 2 liver samples were from Nunavik, QC. The ringed seal samples accounted for 8 positives. Seven of the positive samples were from Sanikiluaq, NU, 6 were blood and 1 was liver. There was also one positive ringed seal liver from Nunavik, QC. Beluga accounted for 7 positive samples, 5 were blood samples and 2 were livers from Hendrickson Island, NT. The positive samples had their viral titers calculated based on the ddPCR quantification. These were then extrapolated to the viral titer of the entire isolated RNA samples that were tested (Table 10).

Table 10. HEV viral titer of ddPCR positives. Test to detect HEV using ddPCR to detect a region in the ORF3 of the HEV genome. Viral titer of the total extracted RNA is shown. The samples were tested in duplicate.

Sample Name	Type of sample	Location	Genome copies /uL of RNA	Genome copies in total RNA
BMH-GRDI-085	Caribou Serum	Northern Ontario	3.22	193.20
BMH-GRDI-090	Caribou Serum	Northern Ontario	3.68	220.80
BMH-GRDI-103	Caribou Serum	Northern Ontario	4.14	248.40
BMH-GRDI-111	Caribou Serum	Northern Ontario	5.06	303.60
BMH-GRDI-148	Caribou Serum	Northern Ontario	2.76	165.60
BMH-GRDI-232	Caribou Liver	Nunavik, QC	3.43	205.99
BMH-GRDI-234	Caribou Liver	Nunavik, QC	0.81	48.36
BMH-GRDI-283	Beluga Blood	Hendrickson Island, NT	2.85	285.20
BMH-GRDI-353	Ringed Seal Blood	Sanikiluaq, NU	15.59	1559.03
BMH-GRDI-362	Ringed Seal Blood	Sanikiluaq, NU	10.11	1011.11
BMH-GRDI-532	Ringed Seal Liver	Nunavik, QC	2.85	170.71
BMH-GRDI-541	Ringed Seal Liver	Sanikiluaq, NU	7.00	419.72
BMH-GRDI-542	Caribou Serum	Northern Ontario	2.07	124.20
BMH-GRDI-543	Caribou Serum	Northern Ontario	1.29	77.28
BMH-GRDI-548	Caribou Serum	Northern Ontario	1.29	77.28
BMH-GRDI-1295	Ringed Seal Blood	Sanikiluaq, NU	1.54	153.69
BMH-GRDI-1298	Ringed Seal Blood	Sanikiluaq, NU	1.71	171.00
BMH-GRDI-1317	Ringed Seal Blood	Sanikiluaq, NU	1.60	160.39
BMH-GRDI-1319	Ringed Seal Blood	Sanikiluaq, NU	6.27	627.13
BMH-GRDI-1329	Beluga Blood	Hendrickson Island, NT	29.04	2904.18
BMH-GRDI-1331	Beluga Blood	Hendrickson Island, NT	64.79	6478.99
BMH-GRDI-1336	Beluga Blood	Hendrickson Island, NT	3.72	371.87
BMH-GRDI-1338	Beluga Blood	Hendrickson Island, NT	5.16	515.90
BMH-GRDI-1354	Beluga Liver	Hendrickson Island, NT	3.28	196.78
BMH-GRDI-1356	Beluga Liver	Hendrickson Island, NT	1.92	115.11

3.5 *Toxoplasma gondii* Magnetic Capture and qPCR

3.5.1 Artificially Contaminated Chicken Breasts

These experiments were done to test the Magnetic capture qPCR method with a controlled number of *T. gondii* oocysts. Oocysts were diluted in series from 10^6 oocysts per mL to 10^2 oocysts per mL and then transferred to cut chicken breast purchased from a retail grocery store (refer to section 2.6.1 artificially contaminated chicken). The chicken samples were spiked with one mL of oocyst dilution per 10 g of chicken breast. The limit of detection for these samples was 10^4 oocysts/mL on 10 g of chicken (Table 11).

Table 11. Magnetic Capture qPCR test on spiked chicken breasts. Test for the limit of detection of *T. gondii* oocysts in spiked chicken breast. The test targeted the 529 bp repeat sequence of *T. gondii*. Average Cq values were calculated. Samples were tested in triplicate.

Samples tested (no. oocysts)	Average Cq
Oocyst suspension (10^5)	31.62
chicken breast (10^5)	36.49
chicken breast (10^4)	36.42
chicken breast (10^3)	-
chicken breast (10^2)	-
chicken breast (0)	-

The "–"indicates that there was no *T. gondii* detected

3.5.2 Ringed Seal

The magnetic capture method was used on 18 ringed seal tissue samples of ample size for utilizing this method. These ringed seal tissues were selected as enough tissue was available to take 10 g from. These tissues were from ringed seals that were harvested in Nunavik, QC. The samples included muscle tissue, heart, brain and diaphragm. Paired samples (brain and muscle) were available from three of the ringed seals, and these were counted as separate samples. Two of the samples showed positive results by qPCR after the magnetic capture method; these samples were both ringed seal brains that showed higher Cq levels than the positive controls (Table 12). The positive controls that were selected for this experiment included a naturally infected beluga brain and beluga heart that were confirmed to be positive in a previous study (Iqbal et al., 2018).

Table 12. Magnetic Capture qPCR testing on ringed seal tissues. Test for the presence of *T. gondii* oocysts in ringed seal tissues using previously tested beluga tissues as positive controls. The test targeted the 529 bp repeat sequence of *T. gondii*. Average Cq values were calculated.

Sample	Average Cq
St. Lawrence Estuary Beluga Heart	25.01
St. Lawrence Estuary Beluga Brain	26.04
Nunavik Ringed Seal sample #32 (Brain)	37.44
Nunavik Ringed Seal sample #27 (Brain)	37.47

3.6 Genotyping

3.6.1 Hepatitis E Virus

Sequencing attempts were made on the positives found using ddPCR of all the country food samples. These attempts were made using three different primer sets; the first attempt made to sequence used the primers and cycling parameters of Drexler et al. (2012) (Drexler et al., 2012) targeting viral RNA dependent RNA polymerase (RdRp) in ORF1. The second attempt to sequence used the primers and cycling parameters of Baylis et al. (2011) (Baylis et al., 2011) using hemi-nested primers targeting the ORF2 region of the genome. The third attempt to sequence used the nested primers and cycling parameters of Boxman et al. (2017) (Boxman et al., 2017) targeting a different region in ORF2 (Figure 2).

The first attempt used Drexler et al. (2012) (Drexler et al., 2012) primers and was able to capture bands for the positive controls on an agarose gel shown in Figure 3. But this method was unable to capture the same bands for the positive samples. This 370 bp product was then extracted for big dye terminator cleanup and Sanger sequencing.

The second attempt using the Baylis et al. (2011) (Baylis et al., 2011) was able to capture potential bands on agarose gels once again (Figure 4), with the approximate size of 347 bp. These were also extracted for big dye terminator cleanup and Sanger sequencing. These bands that were captured unfortunately did not yield any high-quality sequences.

The final attempt using the Boxman et al. (2017) (Boxman et al., 2017) primers yielded similar results to the previous two attempts. The agarose gels were able to capture DNA bands but not at the appropriate size for HEV (Figure 5). Seqeuncing was conducted on the bands that were present on the agarose gel resulting in species specific sequence.

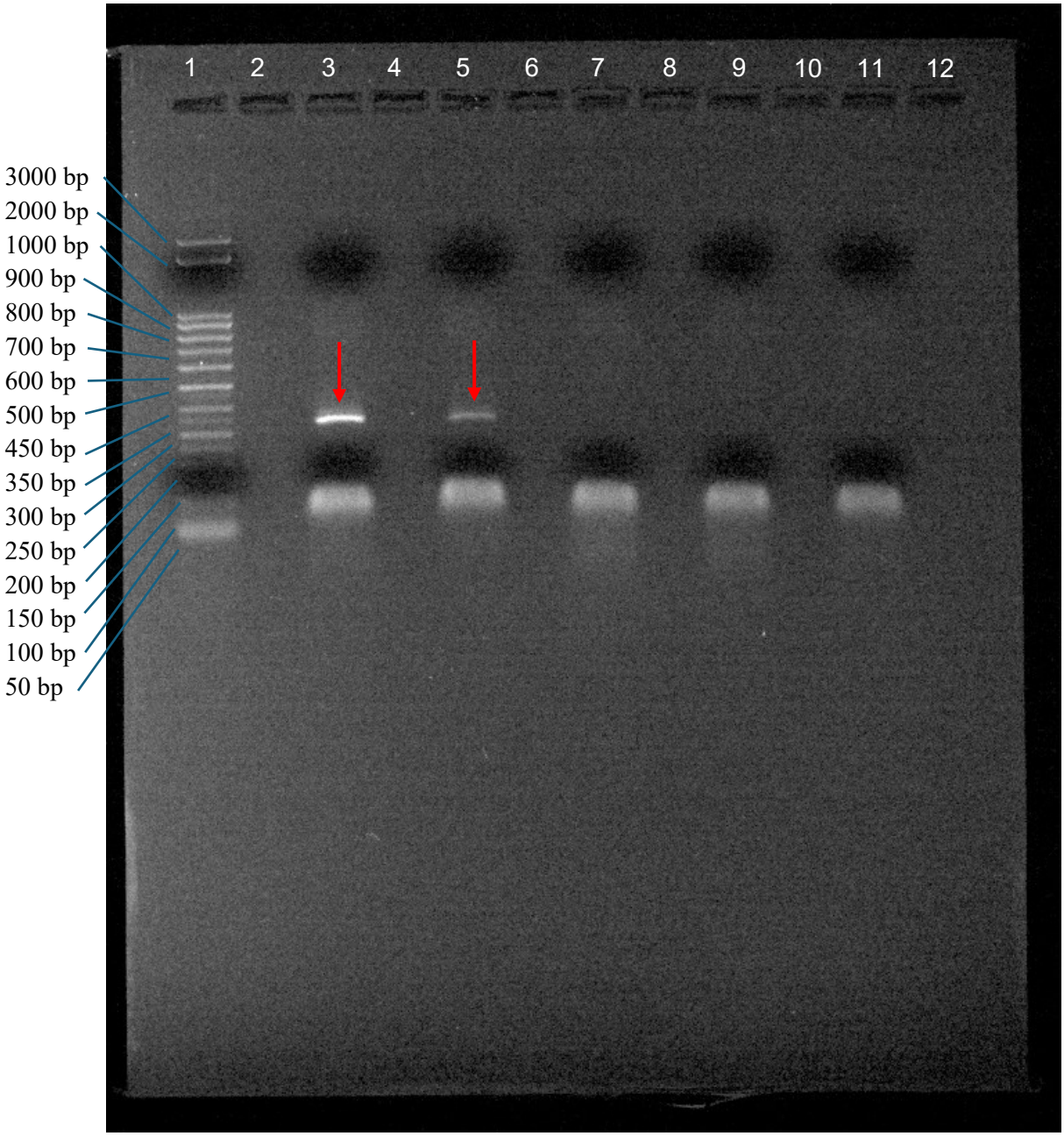


Figure 2. Drexler et al. (2012) primer test on positive control and positive liver samples. This test was using Drexler et al. (2012) primer set and the positive controls in lanes 3 and 5 were HEV positive swine filtrate. Lanes 7 and 9 are the two ddPCR positive caribou liver samples tested were from Nunavik, QC. Lane 11 was a negative control of ultrapure water. Red arrows indicate the excised bands.

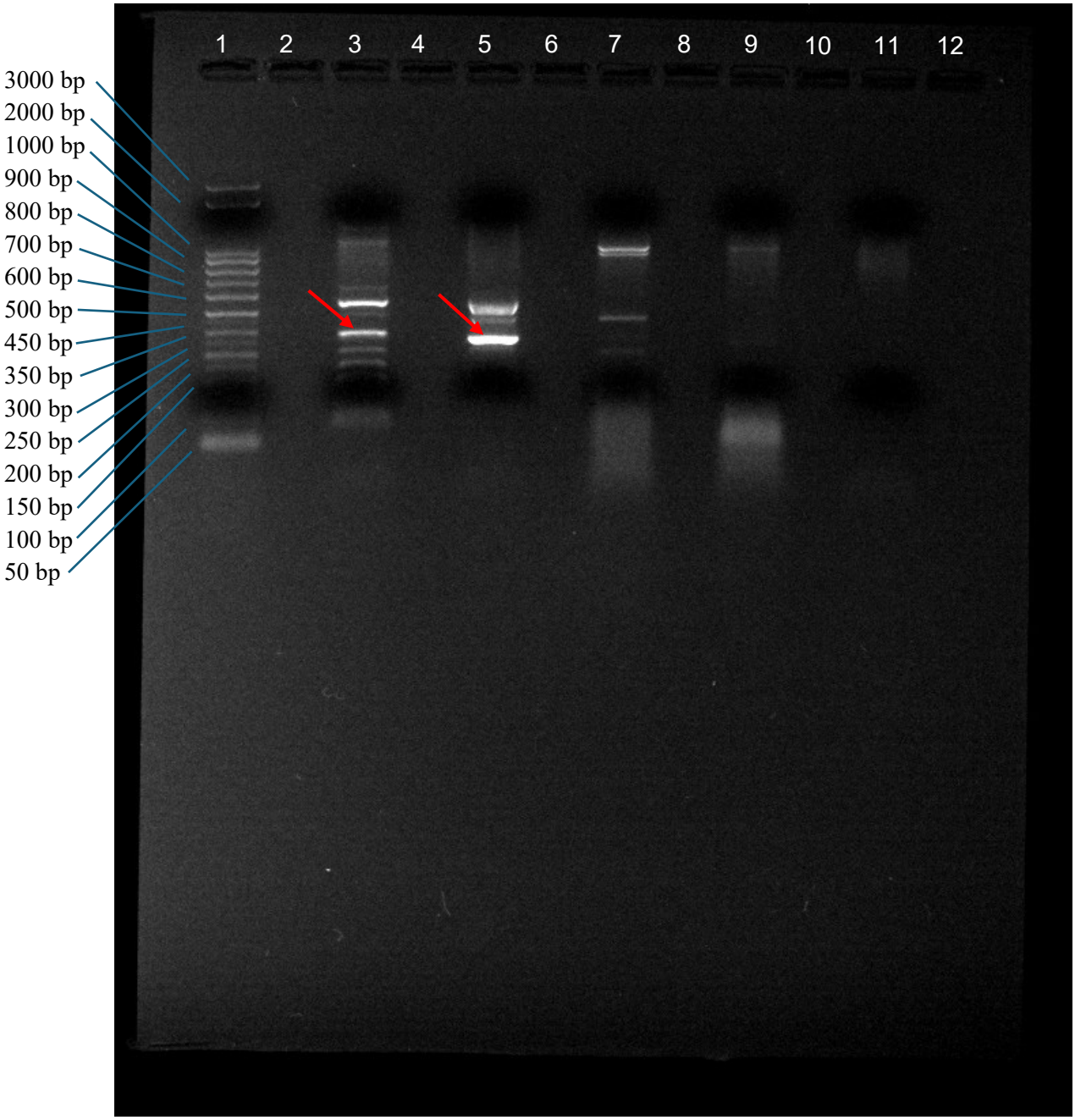


Figure 3. Baylis et al. (2011) primer test on positive control and positive liver samples. This test was using Baylis et al. (2011) primer set and the positive controls in lanes 3 and 5 were HEV positive swine filtrate. Lanes 7 and 9 are the two ddPCR positive caribou liver samples tested were from Nunavik, QC. Lane 11 is a negative control of ultrapure water. Red arrows indicate the excised bands.

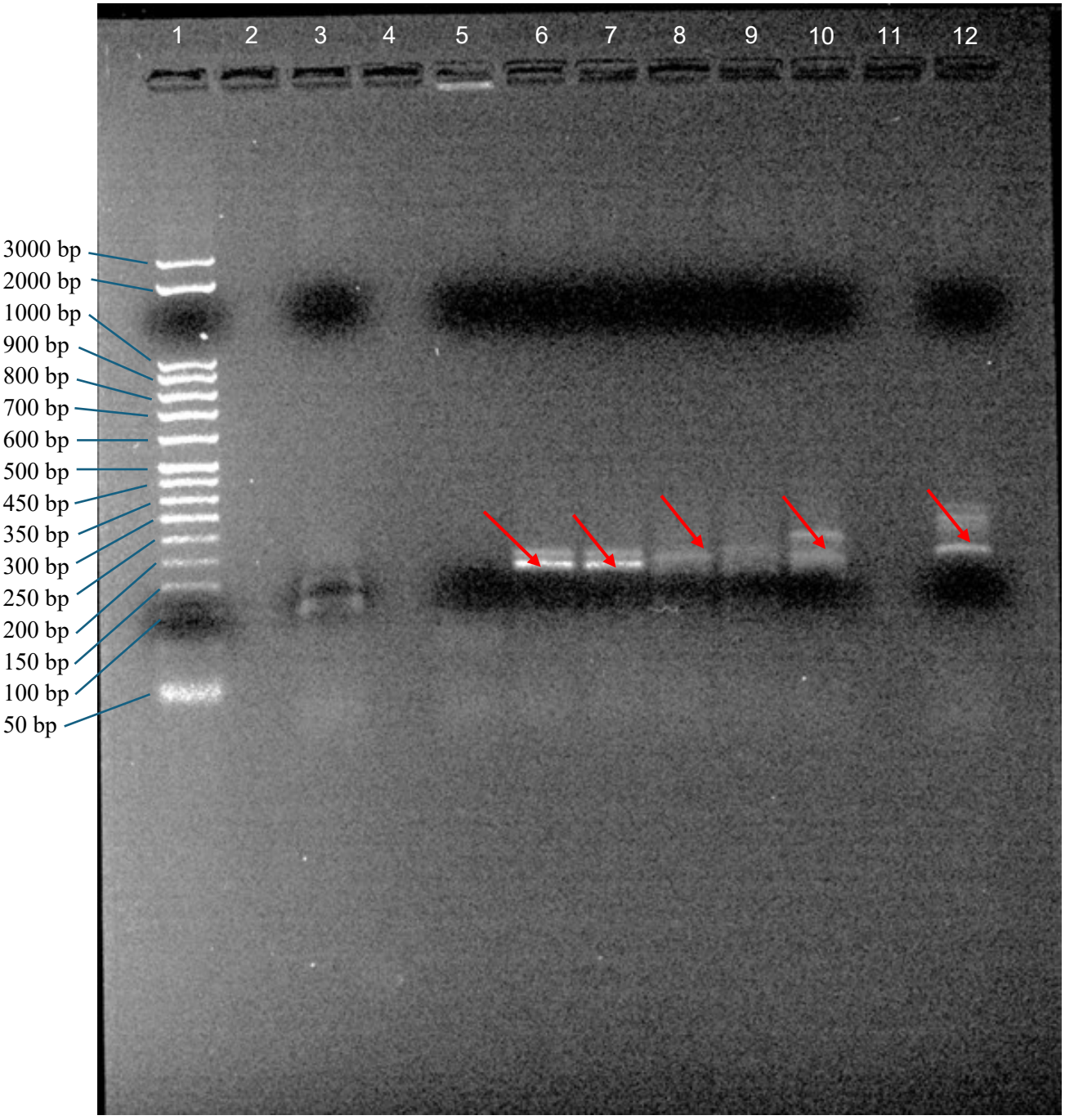
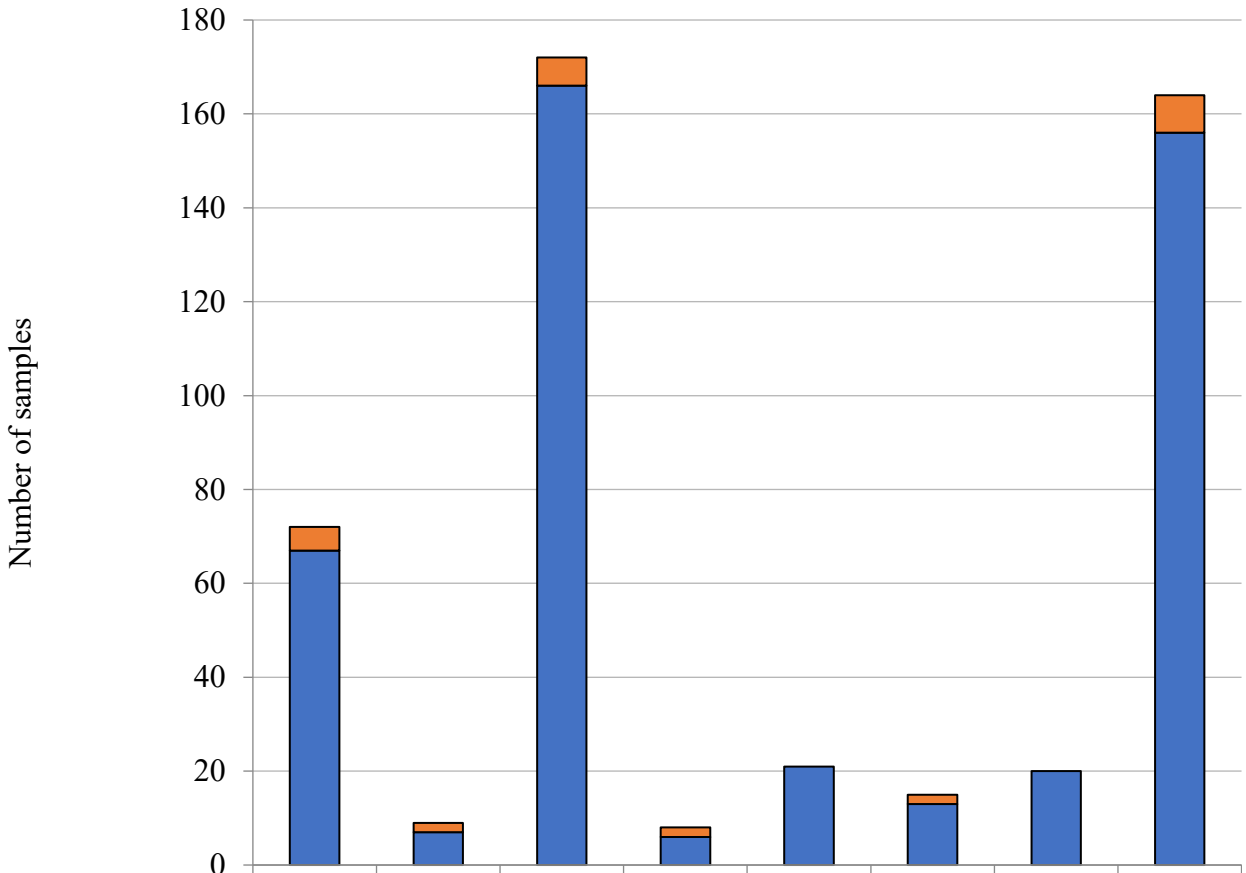


Figure 4. Boxman et al. (2017) primer test on positive control and ddPCR positive samples. This test was using Boxman et al. (2017) primer set and the positive controls in lane 3 was HEV positive swine filtrate. The positive samples were a mixture of caribou serum from Northern Ontario (lanes 8, 9, 10 and 12) and caribou liver from Nunavik, QC (lanes 6 and 7). Lane 5 is the negative control of ultrapure water. Red arrows indicate the excised bands.

Table 13. HEV positive prevalence using ddPCR

HEV positive prevalence results broken down by species and by region. Positives were determined using ddPCR. Each sample was tested in triplicate.

Location	Samples Tested	ddPCR positive	Percent Positive
		Caribou	
Northern Ontario	102	8	7.84
Gods Lake	4	0	0
Pen Island	29	0	0
Cape Churchill	11	0	0
Berens-Sydney Range	36	0	0
Nunavik	35	2	5.71
Unknown	3	0	0
		Ringed Seal	
Arviat	1	0	0
Coral Harbour	14	0	0
Pond Inlet	20	0	0
Sanikiluaq	138	7	5.07
Pangnirtung	1	0	0
Nunavik	6	1	16.67
		Beluga	
East Whitefish	10	0	0
Hendrickson Island	61	7	11.48
Kendall Island	9	0	0
Tuktoyaktuk	1	0	0



	Beluga Blood	Beluga Liver	Ringed Seal Blood	Ringed Seal Liver	Caribou Blood	Caribou Liver	Caribou RNA	Caribou Serum
PCR positive	5	2	6	2	0	2	0	8
PCR Negative	67	7	166	6	21	13	20	156

Figure 5. HEV ddPCR positive results by sample type

HEV positive prevalence results broken down by the type of sample and species. Positives were determined using ddPCR. Each sample was tested in triplicate.

Table 14. *Toxoplasma gondii* positive prevalence using ELISA

Toxoplasma gondii positive prevalence results broken down by species and by region. Positives were determined using ELISA. Each sample was tested in triplicate.

Location	Samples Tested	ELISA positive	Percent Positive
	Caribou		
Northern Ontario	50	1	2
Gods Lake, MB	3	0	0
Cape Churchill, MB	11	1	9
Pen Island, NU	29	0	0
	Ringed Seal		
Arviat, NU	1	0	0
Pangnirtung, NT	1	0	0
Coral Harbour, NU	14	4	28.6
Pond Inlet, NU	21	2	9.5
Sanikiluaq, NU	102	6	5.9
	Beluga		
East Whitefish, NT	10	0	0
Hendrickson Island, NT	33	0	0
Kendall Island, NT	8	0	0
Tuktoyaktuk, NT	1	0	0

Table 15. *Toxoplasma gondii* positive prevalence in Ringed Seal tissue using MC-PCR

Toxoplasma gondii positive prevalence results broken down by sample type. Each sample was tested in triplicate.

Type of Sample	Number of Samples	MC-PCR Positive	Percent Positive
Ringed Seal			
Muscle	7	0	0
Heart	1	0	0
Brain	8	2	25
Diaphragm	2	0	0

4. Discussion

This project was aimed to look at the increasing risk that zoonotic pathogens pose to the Northern Canadian population. This population is at higher risk as they consume country foods that are prepared raw and therefore have the risk of transmitting zoonotic pathogens. This study was looking at the prevalence of Hepatitis E virus and *Toxoplasma gondii* in traditional country foods such as caribou, ringed seal and beluga. This data would then be communicated back to the communities in which the samples were hunted. The goal was to see the role that climate change plays in the transmission of these zoonotic pathogens and to see if they correlate with the increase in human seroprevalence in Canada's North. The original goal of this study was to see how the prevalence of HEV and *T. gondii* may have changed over time with the effects of climate change using a large sample size from a variety of regions in Canada's North. This goal changed as access to the decades old, archived samples at the National Wildlife Research Centre (NWRC) was not granted in time upon completion of this study. The new goal of this project was to establish baseline prevalences of HEV and *T. gondii* in country food samples for future studies on the effects of climate change.

4.1 Hepatitis E Virus Prevalence

A 2015 study done by Fearon et al. looked at the seroprevalence of HEV among Canadian blood donors. These blood donor samples were tested for HEV RNA using RT-PCR and for the HEV IgG and IgM using ELISA. This study found that out of 4102 blood donors there were 241 positive for HEV meaning that among these samples there was a 5.9% seroprevalence. Of those samples there were four samples that also tested positive for IgM meaning that they had a recent exposure to HEV (Fearon et al., 2017). They also conducted a case-controlled study to see the cause of exposure and found that 46 % of the anti-HEV positive group had come into contact with

farm animals with 97% of the individuals commonly eating pork products (Fearon et al., 2017). This is not surprising as a study conducted by Li et al. in 2021 found that worldwide roughly 60% of domestic pigs have encountered HEV infection based on serological analysis (P. Li et al., 2021). This study also showed that there was nearly 13% active infection rate in domestic swine and further a 10% prevalence in commercial pork products (P. Li et al., 2021). The reported Canadian prevalence in pork products was estimated between 9% - 10.5% (Mykytczuk et al., 2017; Nantel-Fortier et al., 2016; Wilhelm et al., 2014).

There is a gap in the literature when it comes to reporting the prevalence of HEV in Canada's North. This study looked to fill the gap by screening multiple country food species such as caribou, ringed seal and beluga. These results come from the ddPCR tests that were conducted throughout this study. Droplet digital PCR was selected as it was able to screen all the different mediums of samples and was more accurate than qRT-PCR methods for detection.

Looking at the prevalence in caribou serum using ddPCR (Figure 5) the rate of positivity of HEV is 5.13 % or 8 ddPCR positives out of 156 caribou serum samples. This result is slightly higher than the previously reported prevalence in a 2017 study done by Weger et al. (Weger et al., 2017) which was found to be 3.2 % in caribou. The study done by Weger et al. looked at caribou from multiple different herds during different years including the Bluenose East herd from 2012, the Beverly herd from 2009 and 2012, the Bathurst herd from 2011 and 2012, and the Owl Lake herd from 2009. The Weger et al. study had some limitations as it only tested serum samples and were not able to find any PCR positives for HEV. Whereas in this study multiple different sample types were examined for the presence of HEV and these samples were PCR positive. When looking at Figure 5, liver tissue has a much higher rate of positivity than that of serum or blood therefore it is crucial that liver tissues are examined in future studies for HEV prevalence. It is also important

to note that the samples from this study include caribou sampled from the regions stated in section 2.1.1 Serum and Whole Blood Samples during the years 2011-2024 meaning that climate change may have increased the prevalence of HEV in caribou serum. Another possible reason for higher detected prevalence was the improvement in diagnostic methods used to detect HEV as ddPCR was more reliable and sensitive when compared to qPCR (P. Zhu & Wang, 2016). Therefore, these results should be used as the new baseline seroprevalence for these specific regions of Northern Canada for future studies.

HEV has not previously been characterized in marine mammals and this is a novel finding for this study as shown in Table 13. The prevalence for ringed seal samples is 5.11 % in Sanikiluaq, NU and 6.25 % in Nunavik, QC. The prevalence in beluga samples is 12.06 % in Hendrickson Island, NT. These results are interesting as the virus has never been characterized in these animals previously therefore further observations need to be made to gain a deeper understanding of which genotypes are infecting these marine mammals. These results further demonstrate that HEV-3 has a wide host range.

Looking at Figure 5, the positive prevalences differ again from sample type to sample type with liver tissue having a higher rate of positivity than that of blood or serum samples from the same animal. This was to be expected as the predilection site of HEV is in the liver. It is also important to note that there were many more blood and serum samples than tissue samples which may skew the results. Future studies will need to test many tissue samples to confirm these findings. These are two keystone country food species therefore it is important that marine mammals are now tested as they can possibly be a host for the virus.

It must be considered that all these samples were provided out of convenience for this study. This was the reason for the low number of samples tested for each region as these tissues

were commonly consumed country food. This meant that these tissues were crucial to the nutrition and culture of the communities that provided them for this study. The regions where these tissues were sampled from were commonly hunted areas in Canada's North. Local hunters hunted and harvested tissues from these species to make traditional country foods for their community. The hunters and communities then shared these country food samples out of convenience to be tested in this study.

4.2 Hepatitis E Virus Genotyping

One of the goals for this project was to identify if there were any new potential host species for zoonotic pathogens. The main testing method to detect HEV in the keystone country food species, caribou, ringed seal and beluga was ddPCR. There were 25 total positives as seen in section 4.3 HEV prevalence. After confirming the presence of the virus, conventional RT-PCR was run to allow for the opportunity to sequence and genotype these specific positive samples. As shown in section 3.6 Genotyping there were three separate attempts to sequence these 25 samples.

The first attempt to used the hemi-nested primers and cycling parameters of Drexler et al. (2012) targeting viral RNA dependent RNA polymerase (RdRp) in ORF1. This primer set was selected as they were shown to have broad reactivity with novel HEV strains and this primer set came with the added benefit of more amplification of HEV as these primers were hemi-nested (Drexler et al., 2012). This attempt was unable to provide a sequence as there were no clear bands on the agarose gel for the RT-PCR.

The second attempt used the primers and cycling parameters of Baylis et al. (2011) using primers targeting the ORF2 region of the genome. This primer set was selected as they showed good results when it came to genotyping HEV (Baylis et al., 2011). During this attempt there were

bands on the RT-PCR agarose gel but the after sequencing there were no high quality sequences that were produced using Sanger sequencing.

The third attempt made to sequence used the nested primers and cycling parameters of Boxman et al. (2017) targeting a different region in ORF2. This primer set was selected as it was used by HEVnet as the widely accepted method for PCR detection and sequencing of HEV (Boxman et al., 2017). Later the Boxman et al. (2017) primer set was found to only be applicable for detecting the European genotypes and subtypes of HEV after analysis using NCBI BLAST. This was discovered upon the comparison of the primer Boxman primer and the Canadian HEV subtype genome where it was observed that there were many identities that were not shared in the regions that the primers bound to the Canadian subtype. The subtypes of HEV that are found in Canada differ slightly from those found in Europe. Therefore, as seen on the agarose gel in Figure 3 the bands were much lower than the 493 bp that the primers were designed for. To figure out what these unwanted PCR products were, these bands were sequenced using Sanger sequencing. These sequencing products produced were identified to be species specific genes belonging to caribou and not the HEV genome. The most probable answer to why this occurred is that there was a region of caribou DNA that was binding to the primers causing the product to be created and amplified. For future studies on caribou these primers may not be suitable.

One of the future directions for this study is to create a new primer set that is designed for the Canadian subtypes of HEV. Once that is complete the next steps would be to genotype the positives samples that were detected by ddPCR. Finally, when those steps are complete to conduct whole genome sequencing on the HEV detected in these country food positives.

4.3 *Toxoplasma gondii* prevalence

4.3.1 Seroprevalence

Toxoplasma gondii has been a parasite of interest in Northern Canada for a long time as it has been shown to be an increasing public health concern for the human population that consumes country food. The seroprevalence of *T. gondii* in humans in Canada's North is much higher than that of the population in the rest of Canada. Understanding the transmission pathways for this parasite is crucial to gain an epidemiological knowledge of this pathogen and to help prevent cases and outbreaks that may arise from eating country foods. In this study three keystone country food species: caribou, ringed seal and beluga, were examined for the prevalence of the parasite.

A study done by Hernández-Ortiz et al. in 2023 (Hernández-Ortiz et al., 2023) found the prevalence of caribou in Nunavik to be 18% and the prevalence for ringed seals reported by Health Canada to be approximately 10.7 % (Reiling & Dixon, 2019). ELISA was the primary detection method as the majority of the samples were either blood or serum. Looking at Table 14 the prevalence in caribou is 2 % in Northern Ontario and 9 % in Cape Churchill, MB. The prevalence in ringed seals was 28.6 % in Coral Harbour, NU, 9.5 % in Pond Inlet, NU and 5.9 % in Sanikiluaq. These results are important because they have large variance in the prevalence based on where these samples were sampled. For caribou, the prevalence in Cape Churchill was 4.5 higher than the Northern Ontario counterparts and the same is found in ringed seal prevalence. As in Pond Inlet the prevalence is approximately 1.6 times higher than Sanikiluaq. Whereas Coral Harbour has a prevalence that is approximately 2.5 times higher seroprevalence than what was previously reported. It is important to note that these samples that were given to us were convenience samples, which may limit the ability to declare statistical significance. This is why it is crucial to consistently test every year to understand the prevalence of *T. gondii* in country food.

4.3.2 Magnetic Capture PCR

This magnetic capture method was a crucial selection for this project as *T. gondii* tissue cysts are randomly dispersed throughout brain, skeletal and smooth muscle tissues. This project focused on large keystone country food species such as caribou, ringed seal and beluga. With animals of this size, it is difficult to screen them for the zoonotic parasite, *T. gondii*. Magnetic capture has the ability to sample larger quantities of tissue meaning that there is a higher probability of there being a tissue cyst within the sample. The protocol was outlined by Opsteegh et al. in 2010 (Opsteegh et al., 2010) and was rated to be able to test up to 100 g of tissue. For this study it was modified to 10 g of country food tissue as any larger amount would cause the stomacher bag to become clogged and the digestion product to be too viscous to function for the rest of the protocol.

The development and selection of proper positive controls was crucial to this study as these controls were utilized for the duration of this project and for future projects using this method for *T. gondii* testing. Oocyst spiked retail chicken breasts were selected as an alternate positive control as there was an abundance of retail chicken breast meat that was frozen for use in other projects. The retail chicken breasts were also skeletal muscle tissue therefore they would be a good substitute for the precious country food tissues. The chicken breast was then prepared in approximately 1 cm^3 pieces, similar to those that were sampled from the country food animals. These 1 cm^3 pieces were then spiked with *T. gondii* oocysts at various concentrations. This was the best way to determine the limit of detection for the magnetic capture PCR method as it approximated a naturally infected sample at a known concentration of oocysts, whilst mitigating the loss of country food tissue.

This positive control did come with some limitations as it may not be as accurate as having a tissue cyst in an infected animal. The concern was later addressed as there were multiple frozen

country food samples that were used in previous projects that were confirmed *T. gondii* positive. Two beluga brains and one beluga heart were selected for this validation as they were confirmed *T. gondii* positive and genotyped in a study by Iqbal et al. in 2018 (Iqbal et al., 2018) and they were also traditional country food tissues. These samples were used successfully to detect *T. gondii* DNA by qPCR after the magnetic capture, validating this method. These samples were used as internal controls for the remainder of the tests.

Looking at the data from the magnetic capture there were two ringed seal samples that were positive out of the 18 that were sampled. A breakdown of these results is found in Table 15. The brain samples had the highest rate of positivity at 2 out of the 8 or 25 %. This was to be expected as one of the known predilection sites for the parasite. This was a reported prevalence of 11.11 % which is higher than previously reported prevalence for ringed seals (Reiling & Dixon, 2019). It must be noted that there were only 8 samples that were tested for *T. gondii* which could skew the results. Future studies need to test more keystone country food species brain tissue to confirm these results. This method is better for testing as it was able to detect *T. gondii* at a higher prevalence than previously reported using qRT-PCR methods. This was most likely due to the use of larger sample sizes and therefore should be the new standard for testing for the parasite in future studies.

4.5 Reporting and Relaying Data

Zoonotic and foodborne pathogens are of great interest to the people who live in Canada's North as their diet largely includes traditionally prepared country foods. These country foods are prepared raw, and this does not kill the zoonotic pathogens that they may contain. Therefore, this study may bring further insight into what different or new pathogens may emerge as a result of climate change. As the climate changes it brings original hosts of both *T. gondii* and HEV in contact with new reservoirs and intermediate hosts that potentially can carry these pathogens.

These animals then will migrate northward to later be hunted by the Northern Canadian population that rely on these animals for food. These country foods are of great cultural significance to these people, and it is difficult to tell them to prepare these foods in a different way. This is why the results of this study will be communicated to the populations that provided these samples along with any scientists in these regions. This will allow for a deeper understanding of both *T. gondii* and HEV transmission, what animals can potentially carry these pathogens and the risk that they pose to the most susceptible populations, i.e. pregnant women or immunocompromised individuals. The hope is that this study will decrease the risk of any public health outbreaks or infections with these zoonotic pathogens by informing the communities of the risks and of the ways that transmission can be mitigated.

5. Conclusion

5.1 Conclusions

Zoonotic pathogens are becoming an increasing concern for Canada's North and that is seen in the increased prevalence in humans of these foodborne pathogens. This study aimed to look at two of these pathogens and how climate change has affected the transmission pathways of *T. gondii* and HEV. This study was able to give baseline prevalences of *T. gondii* and HEV in keystone country food species in Canada's North. This led to the discovery of HEV in two new potential hosts for the virus, ringed seal (*Pusa hispida*) and beluga (*Delphinapterus leucas*). The detection method for HEV used in this study was published and now can be used as the standard for foodborne viral RNA detection using ddPCR. It is also important that liver tissues are tested for future studies in detecting HEV as that was where the highest prevalences were seen in this study. The prevalence of *T. gondii* in these keystone species was generally higher than previously reported. These results will be communicated to the communities in Canada's North with the goal of lowering the risk factors for transmission of foodborne pathogens along with giving these communities a deeper understanding of any new or existing pathogens that may be in country food.

5.2 Future Directions

Some future directions for this study would be to continue the surveillance of these different keystone country food species along with adding other species to the sampling pool. This would allow for more data on these specific zoonotic pathogens and how many other potential hosts there may be for *T. gondii* and HEV. There should also be more zoonotic pathogens added to what was tested for in this study, allowing for deeper understanding of what pathogens the Northern Canadian population comes into contact with therefore reducing the risk of future outbreaks and infections. In order to see how climate change has affected these pathogens the baseline

prevalences should be tested against older samples to see how they have changed over time. This would allow for a deeper understanding of how climate change has affected the transmission patterns of these pathogens. For future studies testing for *T. gondii*, the new magnetic capture qPCR method should be used as the standard when testing country food samples as it is able to detect a higher prevalence in tissue samples than the previously reported methods. One final direction is to successfully sequence the HEV in marine mammals. As these are novel findings, it is necessary to figure out what genotypes of the virus are infecting marine mammals. This may lead to further discoveries of where these marine mammals contracted HEV from and the other potential unknown hosts that can carry HEV.

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