Serological and molecular epidemiological outcomes after two decades of universal infant hepatitis B virus (HBV) vaccination in Nunavut, Canada

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Abstract

Background: Chronic HBV within the Canadian Arctic is considered endemic (>2% prevalence). To control endemic rates in Nunavut, a vaccination program was initiated approximately 20 years ago, targeted at newborns and grade school students, as an interim catch-up program, such that all individuals born after 1980 are potentially vaccinated. This study investigates the efficacy of these programs and is the first seroepidemiological survey to determine HBV prevalence in Nunavut in the post-vaccination era. **Methods:** Anonymized serum specimens scheduled for destruction following routine medical testing were collected from individuals granting consent. Specimens were tested for antibodies to HBV (anti-HBs, anti-HBc) and hepatitis C virus. Anti-HBc positive samples were further tested for surface antigen (HBsAg) positivity, and HBV DNA was extracted from HBsAg positive samples in order to perform molecular characterization. **Results:** 4802 specimens were collected according to the age distribution of Nunavut, with vaccine age cohort specimens comprising just over half of all collected specimens. Overall anti-HCV+ was 0.55%, with all positivity observed among those aged 24 to 69 years old. Total anti-HBc+ prevalence was 9.40%; however, a 10-fold decrease in the rate of HBV exposure was noted among **those born after 1980 compared to those born before (1.89% vs 20.1%, p<0.001)**. HBsAg positivity was primarily documented in individuals born before 1980 (2.55%), although cases still occurred among the vaccine age cohort (0.21%). HBV subgenotype B5 (HBV/B5), known to be unique among Inuit and Alaska Native people, was the most prevalent genotype observed (82%). Vaccine-based antibody as the sole serological marker was evident in the vaccine age cohort, although the rate of decay with increasing age was much greater than anticipated. **Conclusion:** Nearly two decades after the advent of HBV vaccination in Nunavut, HBV prevalence has decreased to 1.17%, indicating a non-endemic or

low risk prevalence. However, the persistence of infection and a lower than expected prevalence of vaccine-based immunity in the vaccine age cohort will require further investigation to understand the causes and consequences.

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Figure 2: Global distribution of HBV genotypes worldwide. Figure reproduced with permission by John Wiley and Sons, Copyright (2009). Taken from [56].

List of Abbreviations

ALT	Alanine Transferase
Anti-HBc	Antibodies to Hepatitis B Core Antigen
Anti-HBe	Antibodies to Hepatitis B e Antigen
Anti-HBs	Antibodies to Hepatitis B Surface Antigen
cccDNA	Covalently Closed Circular DNA
DNA	Deoxyribonucleic Acid
DPT	Diptheria, Pertussis, and Tetanus
ELISA	Enzyme-Linked Immunosorbent Assay
HAV	Hepatitis A Virus
HBcAg	Hepatitis B Core Antigen
HBeAg	Hepatitis B e Antigen
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HDV	Hepatitis D Virus
HEV	Hepatitis E Virus
IL-10	Interleukin-10

IU	International Units
MHR	Major Hydrophilic Region
mRNA	Messenger RNA
NML	National Microbiology Laboratory
NNI	Nearest Neighbour Interchanges
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PreS	Pre Surface Region
RC	Relaxed Circular
RNA	Ribonucleic Acid
RPM	Rotations Per Minute
SPR	Subtree Pruning Regrafting
tRNA	Transfer RNA
WHO	World Health Organization

Introduction

1. General Background

Hepatitis B virus (HBV) causes an enormous global burden, being the 10th highest cause of death by infectious disease [1]. It is estimated that approximately two billion individuals have been exposed to HBV, 400 million persons are chronically infected worldwide, and 500,000 to 700,000 deaths occur annually due to HBV [2].

The rate of infection by HBV is still alarmingly high despite the availability of a very effective, preventative vaccine, which provides lifelong immunity [3]. Many regions and countries that were deemed high-risk for HBV infection have now run routine vaccination campaigns for almost three decades, and follow-up studies now show significantly reduced levels of chronic HBV infection within vaccination age cohorts in regions which include Thailand, Taiwan, and Alaska, amongst others [4-7].

In the current study, the vaccination program in Nunavut, which has been in effect for almost two decades, is being investigated to ensure reduction of HBV infection within the highly endemic Inuit populated region. Western circumpolar HBV prevalence studies have previously shown high incidence of infection within Alaska, the Northern Canadian territories, and Greenland before vaccination was made available [8-10].

2. Natural History of HBV

2.1 Transmission

HBV is transmitted by human bodily fluids. Horizontal transmission occurs by direct blood-blood contact, sexual transmission and intravenous drug use [1]. Vertical transmission

may account for a high proportion of infections in some regions, where infants born to an infected mother have a 90% risk of contracting an HBV infection, dependent on the mother's HBV replicative state during birth [3]. However, expectant mothers are now screened during pregnancy for HBV, and treatment in the third trimester to hinder active HBV replication is given, in addition to a primary immunization dose given to the newborn within 24 hours of birth, which greatly reduces vertical transmission [11].

2.2 Acute HBV Infection

Acute HBV infection is generally asymptomatic in infants and children, where over 95% of infants and 90% of children up to five years old do not display symptoms. However, approximately 30% to 50% of adolescents and adults display non-specific symptoms which include malaise, poor appetite, nausea and pain, and infection may progress to cause fatigue and jaundice. A fulminant course is extremely atypical and occurs in less than 1% of cases in patients symptomatic with jaundice [12].

HBV targets human hepatocytes within the liver, and causes liver damage by host-mediated immune responses. Initial HBV infection does not always prompt the host innate immune response, allowing HBV to infect host hepatocytes and incubate, which lasts weeks or sometimes months, leading to high viremia [12]. This may explain the high rate of chronicity in infants with immature immune systems, as infected adults can achieve viral clearance by mounting rapid humoral and cellular immune responses, and have a much lower risk of chronicity [13]. As viremia increases during the incubation phase, effector molecules and cells of the adaptive immune response eventually become activated.

The adaptive cellular response is initially activated, followed by antibody production. Host T-cell-dependent immune response begins clearing HBV from the host through non-cytolytic mechanisms. However, this is followed by T-cell-dependent cytolytic immune responses, which prompts onset of disease by causing host liver damage [14]. This is due to the high production of CD8+ T-cells within the liver, which react to HBV epitopes, leading to the destruction of HBV infected hepatocytes [15]. Factors such as CD4+/Fox3p+ regulatory T cells aid to mediate immune function and downregulate effector T cells in order to preserve host liver tissue integrity and function, but may prolong viral clearance [16].

Antibody production by memory B-cells is initiated after effector T-cell activation. The majority of antibody produced against HBV will target the virus' envelope protein, surface antigen (HBsAg). Antibodies to HBsAg (anti-HBs) are produced in order to enhance opsonisation of HBsAg and limit further HBV infection by, for example, reactivated HBV [12].

Acute HBV infection is highly self-limiting, described as elimination of HBsAg from the blood within 6 months [17]. Resolution of an acute HBV infection leaves the host with lifelong protection. However, due to the virus' replicative nature, the host is not cleared of HBV when resolution is achieved; HBV is merely controlled by the host immune system much like a latent infection. Therefore, future reactivation is possible during host immunosuppression [17].

2.3 Chronic HBV Infection

If resolution is not achieved during the acute phase, HBV infection persists as a chronic infection, which is characterized as presence of HBsAg in the blood for over 6 months. Chronic HBV infection is a leading cause of both liver cirrhosis and hepatocellular carcinoma (HCC). A high occurrence of chronic infection is usually caused by vertical transmission, as previously

mentioned, the risk of an infant developing chronic infection after exposure to HBV from their infected mother is 90%. However, risk of chronic infection decreases as host age increases (Table 1) [18]. The decreased risk for chronic infection with age can be associated with a maturing host immune system.

Chronic HBV infection is characterized by four stages: immune-tolerant, immune-active, inactive, and HBsAg-clearance [19]. The first phase, the immune-tolerant phase, may last 1 to 4 decades in perinatally infected individuals, as the virus is not recognized as foreign. But this phase is short-lived or absent within infected children and adults. It is characterized by the presence of HBV 'e' antigen (HBeAg), high serum HBV DNA levels (>20,000 IU/ml), normal alanine transferase (ALT) levels, and little to no liver inflammation [18]. Prognosis is favorable during this phase, however, progression to cirrhosis has been reported [20].

The immune-active phase is characterized by presence or absence of HBeAg, high HBV DNA levels (>2000 IU/ml), elevation in ALT levels, and active inflammation [21]. Immune-mediated lysis of infected hepatocytes by increased T-cell response occurs, leading to flares of aminotransferases. These flares may lead to antibodies to HBeAg (anti-HBe) seroconversion, but not always. Transient decrease in HBV DNA levels without anti-HBe seroconversion or hepatic decompensation may occur. Duration of the immune-active phase and frequency of the flares are correlated with risk of cirrhosis and HCC [22-23].

Inactive and HBsAg-clearance states are characterized by the seroconversion from HBeAg to anti-HBe, low or undetectable serum HBV DNA levels, and normal ALT levels, with mild hepatitis and minimal fibrosis after mounting a strong immune response to suppress HBV

Age at HBV exposure	Risk of chronic HBV infection
Birth	90%
Infancy to 2 years old	50%
2 years old to 5 years old	25%-30%
Older than 5 years old	5%-7%

Table 1: Age specific risk of chronic HBV infection. Risk of chronic HBV infection is much higher at younger stages of life, with a 90% risk at birth. This risk declines as individuals get older, which is associated with host immunity maturation.

DNA [18]. If this state is reached, prognosis is generally favorable, as there is no active virus, which may persist indefinitely [24]. Some patients will eventually lose HBsAg at a rate of 0.5% to 2.0% per year [25-27].

However, inactive carriers may undergo HBV reactivation, either spontaneously or by immunosuppression [28-29]. This phase is characterized by HBeAg negativity/anti-HBe positivity, detectable HBV DNA, raised ALT levels, and continued inflammation [30]. Generally patients in this phase are older and have progressed liver disease as the reactivation phase occurs later in chronic HBV infection and disease progression may lead to cirrhosis or HCC [21].

Treatment for patients with chronic HBV infection may aid in anti-HBe seroconversion and prevention of end-stage liver disease by limiting HBV replication [21]. Both interferon and reverse transcriptase inhibitors are used to treat chronic HBV infection [31-33]. Although there is no cure for HBV infection, these treatments manage infection, preventing viral replication, leading to a lowered viral load and amount of virus in the blood.

3. Molecular Virology of HBV

3.1 Genome

HBV is an enveloped virus containing the smallest DNA genome of 3200 bp which infects humans [34]. The genome is circular and uniquely partially double-stranded, with a full length negative sense strand and variable length positive sense strand, which is extended during viral infection and thought to be prevented from completion due to capsid particle envelopment [35].

HBV produces seven proteins: the large (preS1), medium (preS2), and small surface antigen (HBsAg), core protein (HBcAg), HBeAg, 'X' protein, and the viral polymerase. The genome encodes 4 overlapping open reading frames (ORF): core, surface, 'X', and viral polymerase [35] (Fig. 1). The viral genome encodes for four different messenger RNAs (mRNA): the 3.5kb mRNA which exists in two species; the precore-core mRNA and pregenomic mRNA, the 2.4kb large surface mRNA, the 2.1kb middle and small surface mRNA, and the 0.7kb X mRNA. The pregenomic mRNA codes for core protein, viral polymerase, and progeny HBV genomes. The precore-core mRNA yields HBeAg after posttranslational modifications. The 2.4kb mRNA encodes the large surface antigen protein, and, along with the 2.1kb mRNA, produces the middle, and small surface antigen proteins. The 0.7kb mRNA encodes X protein [35].

The hepatitis B virion has an envelope, with large, medium, and small surface antigen studded along the exterior, and contains the nucleocapsid, which is made up of HBcAg containing the viral genome and polymerase within. HBsAg is generally the target for host humoral immunity, as it is exterior of the virion, and also shed during infection as subviral

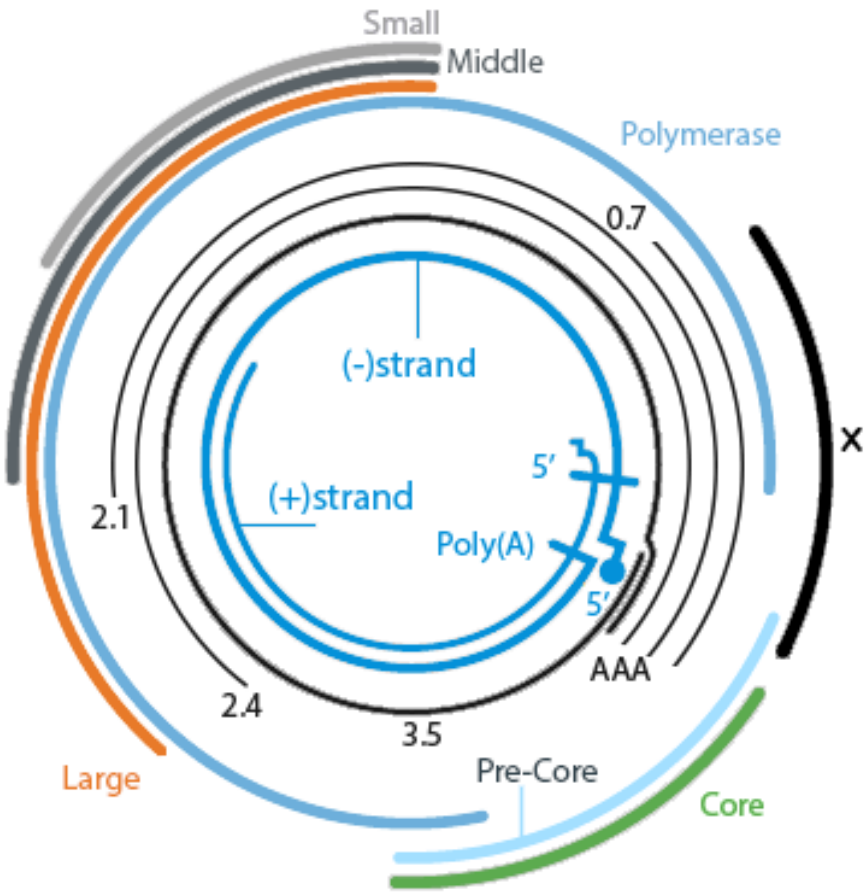

Figure 1: HBV genomic organization. Sites of mRNA transcription and corresponding translational sites for HBsAg (large, middle and small), viral polymerase, core, and X protein.

particles. It contains a major hydrophilic region (MHR) which ranges from amino acid 99 to 169 [36]. The MHR contains a highly conserved region called the ‘a’ determinant, which contains the majority of epitopes for HBsAg [37]. These epitopes are generally the central target for protective humoral immunity against HBV. Other antibodies the host will produce include antibodies to HBcAg (anti-HBc) and anti-HBe. The phase of infection can usually be determined based solely on the viral antigen and host antibody profiles (Table 2).

	Antibody/Antigen Profile			
Interpretation	**Anti-HBs**	**Anti-HBc (Total)**	**Anti-HBc (IgM)**	**HBsAg**
Protected against HBV due to vaccination	Positive	Negative	-----	Negative
Susceptible	Negative	Negative	------	Negative
Naturally Infected	Positive	Positive	------	Negative
Acute Infection	Negative	Positive	Positive	Positive
Chronic Infection	Negative	Positive	Negative	Positive
Unclear, Possibly: - Resolved infection (most common) - False Positive (susceptible) - "Low level" chronic infection - Resolving acute infection	Negative	Positive	------	Negative

Table 2: Interpretation of HBV infectivity state by host antibody profile. By HBV serological testing, the state of HBV infection within a host can be determined by the presence or absence of the HBV seromarkers [38].

Serological detection of antigens and antibodies in an infected patient is most commonly done by commercial enzyme-linked immunosorbent assay (ELISA). The ability to target conserved regions, such as the 'a' determinant of HBsAg, allow for use of commercial kits to identify both antigens (HBsAg, HBeAg) and antibodies to HBV (anti-HBs, anti-HBc, and anti-HBe) in serum for clinical diagnostics.

3.2 Viral Life Cycle

Once in the blood, HBV will circulate until it reaches the liver, where it will attach and infect host hepatocytes. It was recently discovered that HBV interacts with the host hepatocyte receptor sodium taurocholate cotransporting polypeptide via the preS1 domain of the large envelope protein to permit attachment and entry [39]. Once attached, the viral particle will internalize most likely by endocytosis, which releases the viral nucleocapsid, made up of HBcAg [40]. The viral nucleocapsid is transported to the nuclear pore, releasing its contents: the viral polymerase and genome, which is initially in a relaxed circular conformation (RC) [41]. The viral genome is delivered to the host nucleus, where the positive sense strand is repaired, resulting in the conversion of RC to a covalently closed circular DNA (cccDNA) conformation, which is used as the viral transcription template. Various steps are seemingly required for RC to cccDNA conversion, however, these steps remain unclear [42].

Viral mRNAs, once transcribed, are transported to the host cytoplasm for translation and viral replication [42]. To produce viral progeny, pregenomic mRNA must be encapsidated by viral core protein, which self-assemble into the nucleocapsid once the viral polymerase binds a secondary stem-loop structure, epsilon, on the pregenomic mRNA [43-46]. Once attached to epsilon, the viral polymerase will begin to produce the genomic negative-sense strand by reverse transcriptase functionality within the immature core particle. Positive-sense strand synthesis by viral polymerase and pregenomic mRNA degradation by a ribonuclease follows. The encapsidated viral genome may now be enveloped by a lipid envelope containing embedded HBsAg and be secreted from the host hepatocyte [47-49].

The newly synthesized viral genome may have another fate and be transported back into the nucleus to restore the cccDNA pool [50]. Herein lies the difficulty in curing chronic infection, as it is extremely difficult to eliminate cccDNA pools in host hepatocytes' nuclei. cccDNA stably resides within the nucleus unintegrated, in a supercoiled conformation much like a host chromosome or an episome. Thus, even when HBV infection is controlled within the host, reactivation, especially under immunosuppression, is possible.

3.3 Genotypes

Based on viral genomic sequence diversity, at least 10 HBV genotypes have been described (A-J) with several subgenotypes also defined [51]. Genotype divergence is based on greater than 8% nucleotide variance throughout the entire viral genome sequence, and >=4% divergence is observed within subgenotypes [52-54].

HBV genotypes are well characterized for geographic distribution (Fig. 2). Genotype A is prevalent in sub-Saharan Africa (genotype A1), Northern Europe (genotype A2), and Western Africa (genotype A3). Genotypes B and C are highly prevalent within Asia. The majority of genotype B (B2-B4) is found in mainland Asia, while B1 is located in Japan and B5, originally designated B6, is isolated within the western circumpolar region (Alaska, Northern Canada, and Greenland) [55]. Genotype C (C1-C5) is associated with East and Southeast Asia. Genotype D (D1-D6) is prevalent in India, the Mediterranean, Europe and Africa. Genotype E is found in Western Africa. Genotype F (F1-F4) is reported in Central and Southern America. Genotype G causes infection in France, Germany and the United States while genotype H is found in Central America.

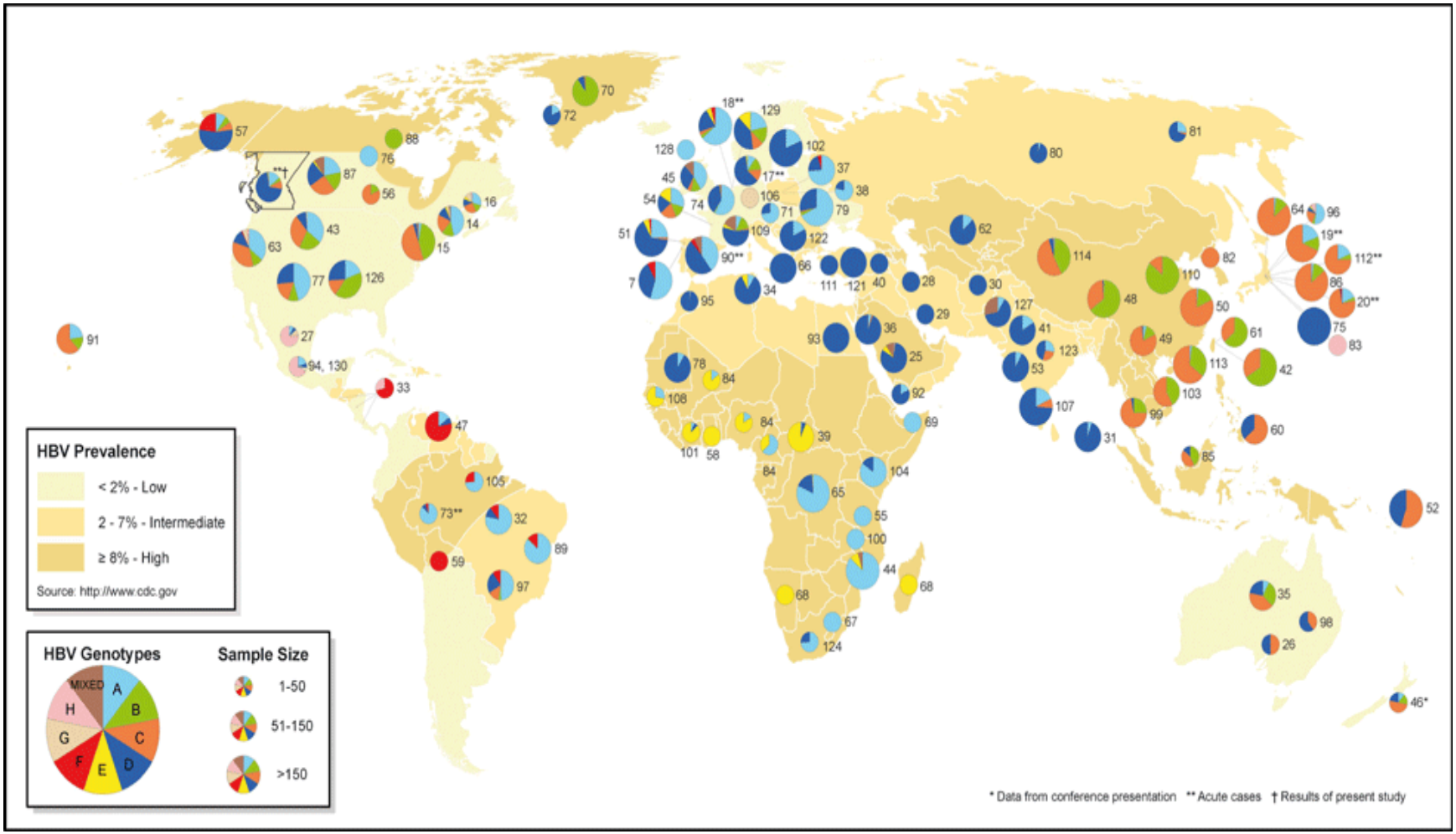

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Figure 2: Global distribution of HBV genotypes worldwide. A map highlighting the different genotypes associated with all regions, globally. As mentioned, genotype B5 is associated with the western circumpolar region while genotypes B1-B4 cause infection throughout Asia.

Two additional genotypes (I and J) have also been described relatively recently. Genotype I is a recombinant strain having some similarity to genotypes A, C, and G, and was identified in Vietnam and Laos [57-58]. Genotype J was isolated in Japan and is closely associated with genotype C [59].

Knowledge of a patient's HBV genotype during infection is important not only for disease progression understanding but also for treatment options. Persistence of an acute HBV infection to chronicity has been shown to be more likely when infection occurs with either genotype A or D compared to genotypes B or C [60-61]. Progression to HCC and cirrhosis has been shown to be more common in genotype C carriers versus genotype B, depending on age, and in patients infected with genotype D or F compared to genotype A [62-64]. Interestingly, subgenotype B5 infection, the dominant genotype studied in this investigation, has a very low incidence for any severe disease outcome, and will be discussed later in this article. Interferon-based treatments were shown to be more effective in reducing HBV infection in patients infected with genotypes A or B compared to those infected by genotypes C or D [65].

4. HBV Epidemiology

HBV prevalence varies across the globe. Generally, developing regions tend to have higher levels of infection and exposure, due to lack of treatment and routine medical assistance. High endemic regions include China, south-east Asia, and sub-Saharan Africa where prevalence of chronic carriers is over 8%, while regions of low endemicity (<2% carrier rate) include North America and western and northern Europe [1].

Canada is not considered endemic for HBV infection with a prevalence estimated to be 0.5-1.0% [66]. Incidence for acute hepatitis B is estimated to be 2.3/100,000, with a higher

incidence in men (3.0/100,000) compared to women (1.5/100,000) [67]. A large portion of cases occur within the immigrant population of Canada, as well as the Inuit and First Nations populations [68-69].

Although the overall prevalence of HBV in Canada is relatively low, the northern territories, comprised of Yukon, the Northwest Territories, and Nunavut, make up a large proportion of chronic HBV infection within the nation. In addition, the surrounding western circumpolar regions of Alaska and Greenland were also highly endemic for chronic HBV infection before vaccine availability. These regions are heavily populated by indigenous populations associated with having high rates of HBV infection.

It has been reported that Alaska Native persons had a high prevalence (6%) of HBV infection, with some communities reaching a prevalence of 20% [9]. Incidence of HCC in Alaska was also high, with one third of all cases occurring in individuals less than 30 years of age with the majority of cases associated with genotype F [64,70].

In 1984, universal infant immunization against HBV and mass population screening for HCC and cirrhosis commenced in Alaska. McMahon et al. showed after 25 years of follow-up, the incidence of acute infection in those aged 20 years or younger decreased from 19/100,000 in 1981-1982 to 0/100,000 in 1993-1994 with no reports of acute HBV infection in children since 1992 [71]. Incidence of HCC within the same age cohort decreased from 3/100,000 in 1984-1988 to 0/100,000 in 1995-1999 with zero cases of HCC since 1999. The overall identification of children 20 years of age or younger positive for HBsAg declined from 657 in 1987 to two in 2008 [9].

Very recently, it has been reported that Greenland is still endemic for chronic HBV with a prevalence of 5-10% in 2011 [72]. However, even with a high endemic rate of HBV infection, incidence of HCC and cirrhosis were not substantially different from Denmark [73-74], which is not considered endemic due to an HBsAg prevalence of 0.1% [75]. Genotype D and B5 are predominant in Greenland, and a high incidence of B5 infection may explain the relatively low occurrence of HCC and cirrhosis [76-77].

Because of the low incidence of end-stage liver disease in Greenland, universal infant vaccination was started very recently in 2010 [72]. Before this, vaccination strategies against HBV were targeted to children born to mothers who were chronically infected. However, after further analysis, the targeted prevention program was not sufficiently effective, whereby failures in identifying children requiring at least 3 doses of vaccination and maintaining proper 'cold-chain' for the vaccines led to decreased efficacy [10].

Like Greenland and Alaska, Nunavut is also considered to be endemic for chronic HBV infection. A seroepidemiological survey was first conducted between 1983 and 1985 in the Northwest Territories of Canada (which included Nunavut at the time) investigating the prevalence of HBV in this region, which greatly consists of Inuit and First Nations inhabitants. Of the 14,198 collected serum specimens, approximately 30% of the total population (51% of the Inuit population, and 44% of the Dene population within the investigated region) were tested, and an overall prevalence of 3% (428/14,198) for HBsAg positivity was found, with some regions reaching 12% positivity [8].

Nunavut officially separated from the Northwest Territories in 1999, which led to a separation in ethnic populations. Nunavut is inhabited by a mostly Inuit population, while the

Northwest Territories is heavily populated with the Dene First Nations population. The division in population helps define HBV genotype epidemiology. Utilizing archived HBsAg positive samples from the serosurvey in the mid-1980s, HBV DNA and molecular genotype analysis was completed. Sixty-nine percent of archived HBsAg positive samples (277/401) were DNA positive. After amplification and sequencing of the viral surface antigen, two major genotypes were found: genotype B and D. An association was discovered whereby all HBV/B infections occurred in individuals self-identifying as Inuit while 91% of HBV/D infections occurred in those self-identifying as Dene. Phylogenetic analysis revealed subgenotypes occurring in this region were B5 associating with the Inuit population, and D3 and D4 associating with the Dene population [78].

Much like Greenland, Nunavut also does not have a high incidence of end-stage liver disease, which may not be expected given the high prevalence of HBV infection. Sakamoto et al. showed in a case-control study investigating clinical differences between genotype B subgenotypes that HBV/B5 causes infection with an inactive, benign outcome, with zero patients progressing to HCC or liver cirrhosis. HBV/B1, the most phylogenetically similar subgenotype to HBV/B5, had 8% of patients progress to end-stage liver disease, while HBV/B2, a 'recombinant' genotype B, had 28% of patients progress to end-stage liver disease [76].

The Sakamoto study then showed HBV/B1 and HBV/B5 clustering together, and apart from the remaining subgenotypes (HBV/B2-B4), phylogentically. HBV/B1 and HBV/B5 can therefore be thought of as 'pure' genotype B, a non-recombinant group, while subgenotype HBV/B2-B4 are a recombinant group, clustering closer with genotype C [76]. It has also been shown that subgenotypes HBV/B2-B4 contain genomic sequence corresponding to HBV/C, which includes the core promoter, precore, and core regions [79].

The increased incidence of end-stage liver disease within patients affected by genotype B2 in comparison to genotype B5 could be explained by the genotype C recombination within the genotype B2 genome. It has been shown that isolates with the double point mutation within the basal core promoter (A1762T/G1764A) lead to a higher frequency of HCC [62]. Because the basal core promoter mutations are generally associated with HBV/C, it could be hypothesized that genotypes B2-B4, containing HBV/C basal core promoter sequence, may lead to increased end-stage liver disease compared to non-recombinant HBV/B. Additionally, the majority of genotype B5 isolates contain the precore G1896A mutation, which is associated with a lower-risk of HCC compared to wild-type G1896 [76,80-81]. Understanding these two separate mutation events could explain the lack of HCC and cirrhosis seen in Greenland's, Nunavut's, and Alaska's indigenous populations, where prevalence and genotype distribution for B5 are relatively high.

5. HBV Vaccination

5.1 History and Dosage Schedule

Preventing infection through vaccination against HBV is arguably the best method in combating infection as treatment only reduces HBV replication but does not cure infection. The HBV vaccine was first approved for use in 1981 in the United States, and in 1992, the WHO recommended integration of HBV vaccination into all national immunization schedules worldwide by 1995 in countries with HBsAg carrier rates of 8% or greater, and by 1997 in all other countries [82-83].

The HBV vaccine is a recombinant component vaccine containing HBsAg produced from yeast cells [84]. The vaccine utilizes a genotype A2 sequence, which has been shown to be

protective across all genotypes, as the majority of protective antibodies are produced against HBsAg within the very conserved ‘a’ determinant region. It has been reported that mutations within the ‘a’ determinant lead to immune escape mutants and breakthrough infections [85].

A full vaccination course generally includes three doses within a given year, and leads to lifelong immunity. For practicality, it was recommended the HBV vaccine be given at the same time the DTP vaccine doses were issued. However, this is problematic as the first dose would not be given at birth. Alternatively, the first dose could be given at birth and the remaining two doses may be administered at the same time the 1st and 3rd doses of DTP vaccine are issued [83].

The importance of receiving the first dose of HBV vaccination at birth is not to be undervalued, especially within endemic regions and countries, as it greatly reduces the potential of perinatal HBV infection. Administration of the first dose of HBV vaccine, along with hepatitis B immunoglobulin, to a newborn infant born to a mother chronically infected by HBV reduces the risk of vertical transmission by up to 90% if given within the first 24 hours after birth, as mentioned previously. This becomes increasingly more important as chronic HBV infection has the highest occurrence early in life compared to adolescence and adulthood.

5.2 Global Use

Currently, 179 countries worldwide have adopted and are implementing a universal infant or adolescent hepatitis B vaccination strategy [86]. However, there are still regions that choose not to universally vaccinate, such as the United Kingdom and the Scandinavian countries, which focus on targeted vaccination against well-defined high risk groups. Although the prevalence of HBV in these regions is not considered endemic, arguments can be made against the targeted

vaccination strategy as it can be difficult to immunize certain high risk individuals such as sex trade workers.

Regardless, it is absolutely necessary for endemic regions to vaccinate at birth once universal vaccination was implemented in order to prevent transmission, especially to infants and children, where chance for persistent chronic HBV infection is highest. Many vaccine program follow-up studies have now been completed, investigating if HBV prevalence for the general population and for the vaccination cohorts within the respective regions have dropped since vaccination has been implemented.

China was a highly endemic region for HBV; a national serosurvey determined HBV prevalence in 1992 was 9.8% in the general population, and 9-12% of children aged 5 years or younger carried HBV [87]. The prevalence was greatly reduced after universal infant vaccination, with presence of HBsAg reduced to 2.1% among all children and 1.0% in children born after 1999 [87]. Prevention in an estimated 16-20 million potential HBV carriers was avoided strictly due to universal infant vaccination [7].

In Taiwan, it was estimated that over 90% of the general population under 40 years of age had come into contact with HBV, and 15-20% of them had chronic HBV infection [88-89]. Between 1984 to 1992, Taiwan first uniquely administered 4 doses of HBV vaccine until switching to the universal 3 dose vaccination afterwards [90]. After 15 years of follow-up, prevalence of HBsAg in subjects 15 years of age or younger had decreased to 0.7% in 1999, with incidence of acute hepatitis B being almost non-existent within the same cohort [5,91].

Similar results have been found in other endemic regions, including Thailand and Alaska, where initial HBV prevalence was 4.3% in children under 18 in Thailand, and 6% in the general

population within Alaska. After follow-up of universal infant vaccination within these regions, prevalence rates of HBsAg within vaccination age cohorts are now 0.7% and almost 0%, respectively [4,6].

In addition to the reduction in HBsAg prevalence, and overall HBV infection, is the decrease in HCC incidence. The HBV vaccine can be regarded as the first anti-cancer vaccine, as inhibiting HBV infection directly prevents possible onset of HCC. In 2002, the number of HCC cases worldwide was estimated to be approximately 625,000 [92]. Although it's not completely understood how HBV causes HCC, studies have shown a large reduction in the number of HCC cases due to vaccination. A study in Taiwan examined diagnosis of HCC in childhood before and after vaccination implementation. Before vaccination, from 1974 to 1984, an incidence of 0.52/100,000 was observed in children who were between ages six and nine. After vaccination, from 1984 to 1986, the incidence dropped considerably to 0.13/100,000 in children of the same age, just two years after universal infant vaccination began [93]. In addition, Alaska has not seen a case of HCC since 1999, as mentioned previously.

The universal vaccination strategy has clearly demonstrated the HBV vaccine's effectiveness at reducing infection in both endemic and non-endemic regions. However, one drawback is becoming more common. HBV exists as a quasispecies within an infected host, and with the ability to immunize against HBV, leads to the selection of escape mutants. As mentioned previously, the 'a' determinant is a highly conserved region of HBsAg, and contains the majority of epitopes that host anti-HBs antibodies will target. Mutations within the 'a' determinant have been observed in persons who have received the HBV vaccine in several regions of the world [4,94-96]. The selective pressure may increase the emergence of surface mutants compared to wild-type. For example, an increase in prevalence of 'a' determinant

mutants from 7.8% to 23.1% was observed in Thailand amongst vaccinated individuals after 15 years of immunization [97]. The emergence of these mutants may lead to breakthrough infections and also may remain undetectable by standard diagnostic methods such as ELISA, which generally detect for wild-type epitopes. However, it's been determined these mutants pose little threat to public health in the near future, and the benefits of universal vaccination still outweigh the drawbacks of the emerging mutants [98].

5.3 Lack of need for a booster

The three dose HBV vaccine has been shown to give lifelong immunity. However, studies have shown that over time, protective anti-HBs levels begin to decay, and can become undetectable or reduced below the protective threshold (<10 mIU/ml), when initial vaccine dose is given at birth (and followed commonly by second and third dose within the first year of life) [71,91,99]. The decaying antibody levels have called into question whether or not the three dose vaccine is sufficient in protecting the host, or if a booster dose is required [100].

Booster studies have been performed to determine if a memory response boosts humoral immunity when the host comes into contact with the pathogen. McMahon et al. demonstrated within eligible persons who received a booster after baseline anti-HBs levels were determined to be <10 mIU/ml after 22 years, that 81% had regained immunity (>10 mIU/ml) after 60 days following booster administration [71]. Su et al. demonstrated, with a similar study after an 18 year follow-up, that 92% of persons who initially had <10 mIU/ml anti-HBs levels regained immunity following a booster dose [91]. Therefore, it could be argued a 4th dose or administration of a booster later in life may not be required. This, coupled with as individuals

grow older, the risk of chronic HBV infection greatly decreasing further decreases the need for an additional dose.

5.4 HBV Vaccination in Canada

Canada is somewhat unique as the universal vaccination program immunizes both infants and grade school children, depending on where the individual lives. Generally, all the provincial regions which are not endemic for HBV, will vaccinate sometime during grade school, except for New Brunswick which, in addition to the Northwest Territories and Nunavut, immunizes at birth. In Yukon, British Columbia, and Prince Edward Island, the first dose is administered at two months of age.

In Nunavut, a much more comprehensive and stringent vaccination program was started in 1995 because of the endemicity of HBV in the region [101]. To prevent the endemic cycle of HBV in Nunavut, a universal infant vaccination was initiated, and in addition to the infant vaccination, two catch-up vaccinations were employed, for children in grade 4 for nine years, immunizing individuals born from 1985 to 1994, and for adolescent individuals born from 1980 to 1984, leading to anticipated full immunization of all individuals born after 1980.

Unlike the majority of Canada where vaccination is issued in grade school, the Nunavut vaccination program aims to administer the first dose of immunization at birth, followed by additional doses at one month, and 9 months of age (Table 3). Vaccine coverage is expected to cover at least 80% of the population according to WHO standards [83].

Vaccination follow-up is not recommended after an individual has received routine immunization through the universal vaccination program. However, follow-up is recommended

for high risk groups which include immunocomprimised individuals, high risk pregnant women, infants born to infected mothers, and health-care workers at risk of occupational exposure [66].

Rationale

After almost two decades of universal vaccination in Nunavut, no follow-up study has been done to determine the effectiveness of the vaccination program. This study will determine vaccination coverage by analyzing the prevalence of protective anti-HBs antibody within the sample population, as well as the prevalence of infection seromarkers, anti-HBc and HBsAg, to determine if Nunavut is still considered endemic for HBV infection. DNA positive samples will also be analyzed to investigate HBV epidemiology in Nunavut, and whether HBV/B5 is still the dominant genotype in this region.

Hypothesis

The hypothesis for this study is two-fold, pertaining to the vaccination program and the molecular epidemiology of HBV in Nunavut. For the vaccination program, it is hypothesized that the overall prevalence of HBV has declined due to vaccination, which will result in an increase in anti-HBs positivity as the sole HBV seromarker within the population, and a decrease in HBV infection seromarkers, anti-HBc and HBsAg. For HBV epidemiology, it is hypothesized that HBV/B5 is still the dominant genotype within this region.

Province/Territory	Time of HBV Vaccination
Alberta	Grade 5
British Columbia	2, 4, and 6 months (Booster in grade 6)
Saskatchewan	Grade 6
Manitoba	Grade 4
Ontario	Grade 7
Quebec	Grade 4
New Brunswick	Birth, 2, 6 months
Nova Scotia	Grade 7 (2 doses)
Prince Edward Island	2, 4, 15 months
Newfoundland	Grade 6
Yukon	2, 4, 12 months
Northwest Territories	Birth, 1, 6 months
Nunavut	Birth, 1, 9 months

Table 3: Canadian provincial and territorial vaccination schedule. The vaccination schedule in Canada is controlled at the provincial and territorial level, hence the large variability of when individuals are vaccinated. Canada utilizes a 3 dose vaccine schedule, however, British Columbia offers a booster dose in grade 6 and Nova Scotia only offers 2 doses [102].

Materials and Methods

Patient Samples

Serum samples were collected from all individuals who had blood drawn for any routine medical testing at all health care facilities in both the Kivalliq and Qikiqtaaluk regions of Nunavut beginning in April 2013 until the end of April 2014. Because samples used in this study were not initially collected for this purpose, ethical approval was required and granted by the University of Manitoba Research Ethics Board (Winnipeg, MB, Canada) and the Public Health Agency of Canada Research Ethics Board (Ottawa, ON, Canada). Review and approval of the study and its ethical considerations were conducted and a license was granted by the Nunavut Research Institute. In accordance with the ethical approval, all individuals eligible for the study were given the option to deny use of their blood sample through an opt-out procedure at the time of medical testing.

Samples from patients who did not opt-out of the study were collected and delivered to the National Microbiology Laboratory (NML) (Winnipeg, MB, Canada) approximately every two to four weeks per each region. Identifiers on every sample were eliminated, with the exception of birth date, gender, and the community the sample was sent from, which were recorded. To eliminate sample bias, all subsequent samples with the same three identifiers as a previously received sample were assumed to originate from the same patient and were destroyed, as it was not uncommon to receive upwards of three to four samples with identical identifiers in a single shipment likely due to multiple blood collection tubes from a single individual.

Serology

The Roche cobas e411 seroanalyzer (Roche Diagnostics. Laval, QC, Canada) was used to test for presence of the following seromarkers: anti-HBs, anti-HBc, HBsAg, and antibodies to hepatitis C virus (anti-HCV).

Presence of anti-HBs, anti-HBc, and anti-HCV were initially tested for. Samples positive for anti-HBc, indicating past or present exposure to HBV, were further tested for presence of HBsAg. Samples positive for HBsAg indicate chronic HBV infection within the patient and HBV DNA testing and analysis would follow.

In order to eliminate discrepancies and confirm initial results after initial serological results analysis, further confirmatory testing was completed. In accordance with Roche cobas anti-HBc testing, a secondary serological anti-HBc test was performed on samples with borderline results. Because this study focused on the HBV exposure reduction within the vaccination cohort, only samples with borderline results and were in the vaccination age cohort (born after 1980) underwent secondary anti-HBc testing.

HBsAg positive/HBV DNA negative samples were tested for HBsAg neutralization, to confirm presence of HBsAg in serum. HBsAg positive/HBV DNA positive samples were not tested by HBsAg neutralization as HBsAg confirmatory testing was not necessary.

Extraction of HBV Genomic DNA

All HBsAg positive samples were extracted for HBV DNA for further analysis. These samples underwent a phenol-chloroform extraction on 150 µl of sera. Sample sera was added to 150 µl of HBV lysis buffer, 20 µl of proteinase K (Invitrogen, CA, USA), and incubated at 65^{o}C

for two hours. After incubation, three washes were performed to lyse viral particles and isolate viral genomic DNA by adding: i) 300 µl phenol ii) 150 µl phenol and 150 µl chloroform, and iii) 300 µl chloroform. Each wash was followed by a 10 minute centrifuge spin (13,000 RPM), and the upper phase was isolated and added to the next wash. After the third wash, the upper phase was added to solution which cleaned and isolated the HBV genome DNA, and contained 960 µl 100% ethanol, 125 µl ammonium acetate, and 1.8 µl tRNA (10 IU/ml), and was incubated at -20^{o}C overnight.

After overnight incubation, samples were centrifuged for 30 minutes (13,000 RPM) and the solution was decanted, leaving a salt pellet containing the HBV genomic DNA. A last wash step was done by adding 1 ml of 70% ethanol, centrifuged for 10 minutes (13,000 RPM) and decanted afterwards. Tubes were left open to allow for evaporation of the remaining ethanol and 25 µl of ddH_2O was added to resuspend the HBV DNA.

Amplification, Sequencing, and Quantification of HBV DNA

HBV DNA was amplified targeting both surface and precore genomic regions using the AmpliTaq Gold PCR System (Applied Biosystems, California, USA) to determine HBV DNA positivity. Nested polymerase chain reaction (PCR) was performed for both targets. First stage primers HBVPr134 (5’ TGCTGCTATGCCTCATCTTC 3’) and HBVPr135 (5’ CARAGACA AAAGAAAATTGG 3’) (409 bp) were used to investigate HBV HBsAg, and were nested with HBVPr75 (5’CAAGGTATGTTGCCCGTTTGTCC 3’) and HBVPr94 (5’GGYAWAAAGGG ACTCAM GATG 3’) (342 bp), if required. To investigate the HBV precore region, ep1 (5’GCA TGGAGACCAC CGTGAAC 3’) and ep2 (5’GGAAAG AAGTCAGAAGGCAA 3’) (368 bp)

were used for first stage amplification and nested with ep3 (5'CATAAGAGGAC TCTTGGACT 3') and ep4 (5'GGC AAAAAAGAGAGTAACTC 3') (306 bp), if required.

Thermocycling conditions for both stages amplifying HBV surface and precore region were the following: initial denaturation at 94^{o}C for 12 minutes followed by 40 cycles (35 cycles for nested stage) of denaturing step at 94^{o}C for 30s, annealing step at 45^{o}C or 55^{o}C for surface or precore amplification, respectively, for 30s, and extension step 72^{o}C for 30s, followed by final extension at 72^{o}C for 5 min. PCR amplicons were run on a QIAxcel (Qiagen Inc., Toronto, ON, Canada) to investigate positivity and DNA positive samples were submitted to the NML DNA core facility for sequencing.

The Realstar HBV PCR 1.0 kit (Altona Diagnostics, Toronto, ON) was used for HBV DNA detection and quantification. Samples were measured against a mean standard curve utilizing kit controls: 10^1 IU/ml, 10^2 IU/ml, 10^3 IU/ml, and 10^4 IU/ml, which were run in duplicate. DNA quantification reactions required 2 µl of reagent master A, 8 µl of reagent master B, 1 µl of internal control, and 10 µl of HBV DNA template. The real-time protocol, performed on a 7500 Fast Real-Time PCR System (Applied Biosystems Canada. Streetsville, ON), was run for 2 minutes at 95^{o}C, then for 45 cycles: 95^{o}C for 15s, 58^{o}C for 30s, and 72^{o}C for 30s. FAM fluorescent with no quencher was used to detect HBV DNA and JOE fluorescent with no quencher detected internal control DNA.

Full Genome Sequencing

A subset of genotype B5 samples with relatively higher viral loads were amplified for full genome sequence analysis. HBV/B5 generally has a lower viral load compared to other genotypes, therefore, amplification of a 3.2 kb full-length HBV DNA product with a

proofreading polymerase required a several step process starting with initial amplification using primers PhenoP1 (5'CCGGAAAGCTTATGCTCTTCTTTTTCACCTCTGCCTAATCATC3') and PhenoP2 (5'CCGGAGAGCTCATGCTCTTCAAAAAGTTGCATGGTGCTGGTG 3') as described previously by Gunther et al. [103]. Amplification parameters for PhenoP1/P2 PCR included: initial denaturation at 94^{o}C for 2 minutes, followed by 35 cycles of denaturing at 94^{o}C for 30s, annealing at 55^{o}C for 30s, extension at 68^{o}C for 3 minutes, and then a final extension at 68^{o}C for 10 minutes. During the 35 cycles, the extension time was increased by 10s per cycle for the last 25 cycles.

This amplification was not sensitive enough to obtain a product for sequencing, but instead was used as a template for further nested amplification. Six primer sets were used to amplify (Table 4), allowing for "stepwise" amplification and sequencing of the full genome. Utilizing 2 µl of template from PhenoP1/P2 amplification, PCR parameters for both 1st stage and nested reactions were identical as surface and precore reactions described earlier, using the annealing temperatures found in Table 4. If nested PCR did not yield a product, 1st stage and nested stage were performed using the direct DNA extract of the respective samples.

HBV DNA Sequence Analysis

Lasergene sequence analysis software (v10.0; DNASTAR Inc., WI, USA) was used to analyze HBV DNA sequences. Mutations could be analyzed using this software package while genotyping and subgenotyping were done phylogenetically through alignments with GenBank reference sequences using ClustalX (v2.0.10) and MEGA (v5.2) [104,105].

Once alignments were complete for surface, precore, or full genome sequences, a phylogenetic tree was constructed for the respective alignments. DIVEIN online software was

Primers:	Annealing Temp.:	Primer Sequences (5' to 3'):	Region of Amplification:	Expected Product Size (bp):
1st stage: LLf LLr Nested: nLLf nLLr	55°C 55°C	TCCTGCTGGTGGCTCCAG CGTTGACATACTTTCCAATCAA ACCCTGYRCCGAACATGGA CAACTCCCAATTACATARCCCA	Surface	914 760
1st stage: SYBR51HBV FLG2 Nested: Xf07 Cpr	50°C 58°C	GCTGACGCAACCCCCACT GTTGCATGGTGCTGGTC CTCCTCTGCCGATCCATACTGCGGAACTCC CCAATTTATGCCTACAGCCTCCTA	X	634 534
1st stage: HBVLA2 C2R Nested: survCF survCR	60°C 45°C	ACCTCTGCCTAATCATCTCATGTTCATGTC YCCCACCTTATGWGTCCAAGG GGCTTTRGGGCATGG GACGCGGYGATTGAG	Precore/core	593 530
1st stage: PS104 preS1rev Nested: PIS104 preS1prR	45°C 45°C	CCCTATCTTATCAACACTTCC CCTGAACTGGAGCCACCAGCAGG TTCCGGATCCTACTGTTGTTA CTTAGAGGTGGAGAGATGGG	Pol/PreS	989 863
1st stage: PS102 SYBR52REV Nested: PIS102 ENH1Rev	55°C 55°C	CCTATTGATTGGAAAGTATGTCAA AGGAGTTCCGCAGTATGG CGTATTGTGGATCCTTTGGGTTT CTCCAGACCGGCTGCGAGC	ENHI/X/ Precore	313 287
1st stage: PA100 PS100	55°C	GAAGTCCACCACGAGTCTAGA ATCCTCAGGCCATGCAGT	Pol/PreS/ Surface	288

Nested: PIA100 PIS100	55°C	GGTATTGTGAGGAAGCTTGTC CCACCAAACTCTTCAGGATCC		227

Table 4: Primers used for step-wise full genome sequencing. Primer sets used to amplify the full genotype B5 genome for sequencing and phylogenetic analysis. 2 µl of PhenoP1/P2 amplicon was used as the template for the1st stage. If 1st stage amplification was not sufficiently sensitive for detection, a nested stage was run using 2 µl of 1st stage amplicon. Surface and precore sequences used for HBV DNA detection and genotyping were also used for full genome sequencing.

used to construct the trees with the Kimura 2-parameter plus gamma substitution model for the surface tree, the Kimura 2-parameter plus gamma and invariant substitution model for the precore tree, and the general time reversible plus gamma and invariant substitution model for the full genome sequence tree. The substitution model used, including gamma and invariant parameters were defined by MEGA's best fit model output, and the gamma and invariant parameters for phylogenetic tree construction were 0.25, n/a; 0.45, 0.36; 0.63, 0.34 for surface, precore, and full genome trees, respectively. The maximum likelihood phylogenetic trees produced using DIVEIN were done with 100 bootstrap replicates and the BioNJ algorithm for tree construction. Both nearest neighbour interchanges (NNI) and subtree pruning and regrafting (SPR) algorithms were used for optimizing tree topology and branch length [106].

Statistical Analysis

A 2-tailed, 2 sample proportion calculation was used to determine the sample population size required to estimate the level of population protection based on the WHO recommendation of at least 80% as a vaccination coverage target. χ^2 test was used to determine if changes in HBV seromarker prevalence from the non-vaccinated to vaccinated cohorts were statistically significant.

Results

Patient Samples

Samples were received at the NML from Nunavut, Canada from April 2013 until the end of April 2014. Four thousand eight hundred and two samples were received from 19 different communities within Kivalliq and Qikiqtaaluk. One thousand seven hundred and twenty-one (35.8%) of the sample population were from male patients and 3081 samples (64.2%) were from female patients. Nunavut's actual population is divided as 51.8% male and 48.2% female [107]. Median age of the sample population was 29 years old and Nunavut's actual population's median age is 25 years old. Age range of the sample population ranged from 1 week of age to 93 years.

The sample population for this study was separated into two major cohorts for analysis, the presumed non-vaccinated and vaccinated populations, based on the individual's age when the specimen was received (Table 5). Twenty-eight received samples did not have sufficient serum for testing, leaving 4774 samples for HBV serological testing. Determining universal vaccination efficacy and, subsequently, a possible reduction of HBV infection rates in the population due to vaccination was based on the interpretation of the serological differences between the two cohorts.

The received sample population was initially intended to mimic Nunavut's population by age stratification, in order to determine the most accurate representation of the population. Age stratified sample collection was met (Fig. 3) with the exception of the two youngest age groups (0 to 9 and 10 to 19 years), likely due to two factors; the very high population size of those age groups relative to other age groups in Nunavut, and the relatively small number of individuals in these age groups who visit clinics or hospitals for a blood testing procedure.

Cohort	Age	Total number of samples	Total number of samples tested for anti-HBs as sole marker	Total number of samples tested for anti-HBc
Total samples	---	4774	4771	4766
Non-vaccinated	34 years old and older	1965	1965	1963
Vaccinated	33 years old and younger	2809	2806	2803
- Infant vaccination	<1 years old to 18 years old	937	934	933
- Grade 4 catch-up	19 years old to 28 years old	1380	1380	1379
- High School catch-up	29 years old to 33 years old	492	492	491

Table 5: Vaccinated and non-vaccinated cohort sample numbers based on age at received date. A breakdown of the two major cohorts for the current study based on age; samples with ages of 33 years old and younger were included in the vaccination cohort with the assumption that in 2013, those born in 1980 or after were part of the vaccination cohorts, and any samples with ages 34 years old and older were included in the non-vaccinated cohort. The vaccination cohort was also separated into the three vaccination cohorts (infant, grade 4 catch-up and high school catch-up) for further analysis. Three samples positive for anti-HBs were excluded from "anti-HBs only" result because presence of other HBV infection seromarkers were not tested for due to insufficient serum. Eight samples were excluded for anti-HBc testing from the 4774 total samples due to insufficient serum quantities.

The WHO recommends at least 80% of all infants in a population have 3 doses of HBV vaccine [108]. This estimated coverage is based on vaccination records; however, as a surrogate measure of the Nunavut population, the prevalence of anti-HBs, as the sole serological marker, will be determined. To determine the population size required for the current study to be statistically significant, a 2-tailed, 2 sample proportion calculation was used, with parameters 0.8 as proportion 1, an alpha of 0.05, and beta of 0.2. Proportion 2 was calculated at both 0.75 and 0.85 levels. These proportions were selected in order to have a sufficient sample to detect as low as 5% difference between the sample set and the target 80% set, allowing for detection of any differences larger than 5%. It was determined a population of 810 samples (power = 99.9%)

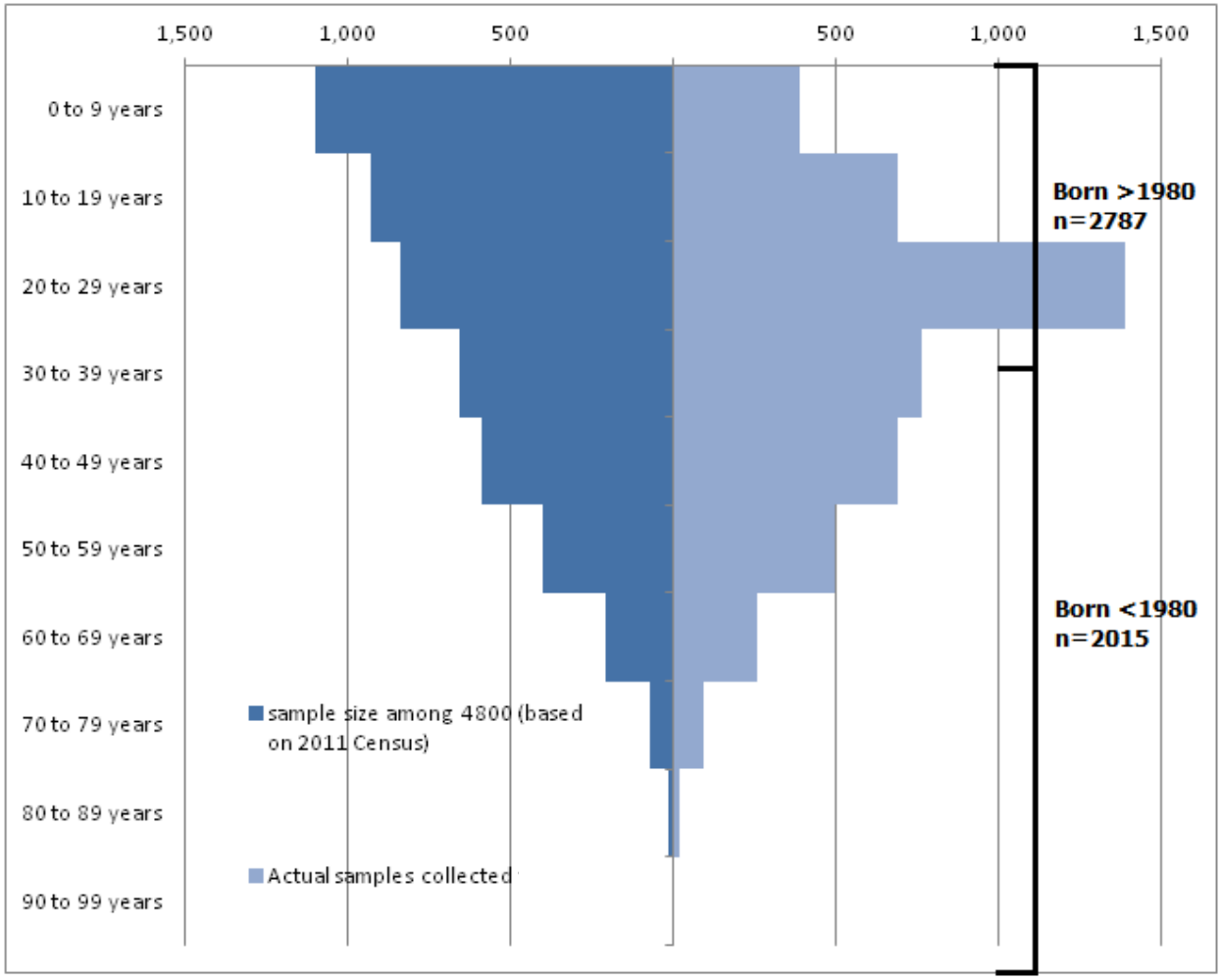


Figure 3: Age stratification of Nunavut population and the sample population. A graph comparing the 2011 population based on census data, approximated to 4800 samples, and the actual received samples for the current study [109] The collected sample used for the current study was intended to replicate Nunavut's actual population based on age stratification. Target sample sizes for each age group were met with the exception of the two youngest groups (0 to 9 years and 10 to 19 years). This was most likely due to the very large proportion of these age groups, compounded with the unlikeliness of individuals within this age group having blood tested, compared to older age groups.

within the universal infant vaccination cohort was required, and which was surpassed by the current study.

Prevalence of vaccine-based protection

A total of 4774 samples were tested for quantitative anti-HBs antibody as the sole serological marker to estimate the prevalence of vaccine-based protection in Nunavut. Prevalence within the entire sample population with protective anti-HBs levels as the sole HBV seromarker in Nunavut was determined to be 23.5% (1119/4771) (Fig. 4) (Table 6). Three positive anti-HBs samples were excluded in the analysis (from the total 4774 samples) due to insufficient serum for further testing of other HBV seromarkers. Thus, without further testing and results, presence of other seromarkers could not be ruled out. Within the vaccination cohort, 28.7% (804/2806) of individuals had protective anti-HBs levels and 16.0% (315/1965) within the non-vaccinated cohort had protective anti-HBs levels. Individuals from the non-vaccinated cohort most likely have anti-HBs antibody as a sole seromarker due to immunity from infection (rather than vaccination) and anti-HBc was lost as a marker of immunity.

The vaccine cohort's anti-HBs positivity for infant, grade 4 catch-up, and high school catch-up cohorts is as follow: 17.6% (164/934), 33.8% (466/1380), and 35.4% (174/492), respectively.

HBV Exposure and Chronic infection

Specimens were also tested for anti-HBc, the seromarker indicative of a past and/or present infection (Table 6). Eight samples were excluded from anti-HBc testing due to insufficient serum.Note that 5 of the 8 samples were anti-HBs negative and so were still included

in the anti-HBs testing totals for determination of anti-HBs only prevalence. A prevalence of 9.40% (448/4766) was found in the entire sample population, but a 10-fold decrease between the non-vaccinated and vaccinated cohort could be observed. The

non-vaccinated cohort had a prevalence of 20.1% (395/1963) for anti-HBc, and the vaccinated cohort had a much lower prevalence of 1.89% (53/2803) ($p<0.001$).

The incidence of exposure was relatively similar throughout the different cohorts of the vaccination program. Those vaccinated in infancy had a prevalence of 2.04% (19/933), the individuals vaccinated in grade 4 had a prevalence of 1.60% (22/1379), and the high school catch-up program cohort had a prevalence of 2.44% (12/491).

Samples positive for anti-HBc were then tested for HBsAg positivity, which is indicative of a chronic infection. Fifty-six anti-HBc positive individuals were HBsAg positive, leading to an estimated HBsAg prevalence of 1.17% (56/4766) within the entire population (Table 6). The majority of HBsAg positive individuals were born outside of the vaccination age cohort (2.55%; 50/1963), with the vaccination cohort having 0.21% seropositivity (6/2803) ($p<0.001$).

Two HBsAg positive samples were from individuals presumed to be vaccinated at birth (3 and 18 years old). Without sufficient background information or vaccination records, it is unclear whether these individuals received a full 3 dose HBV vaccination or if breakthrough infections occurred.

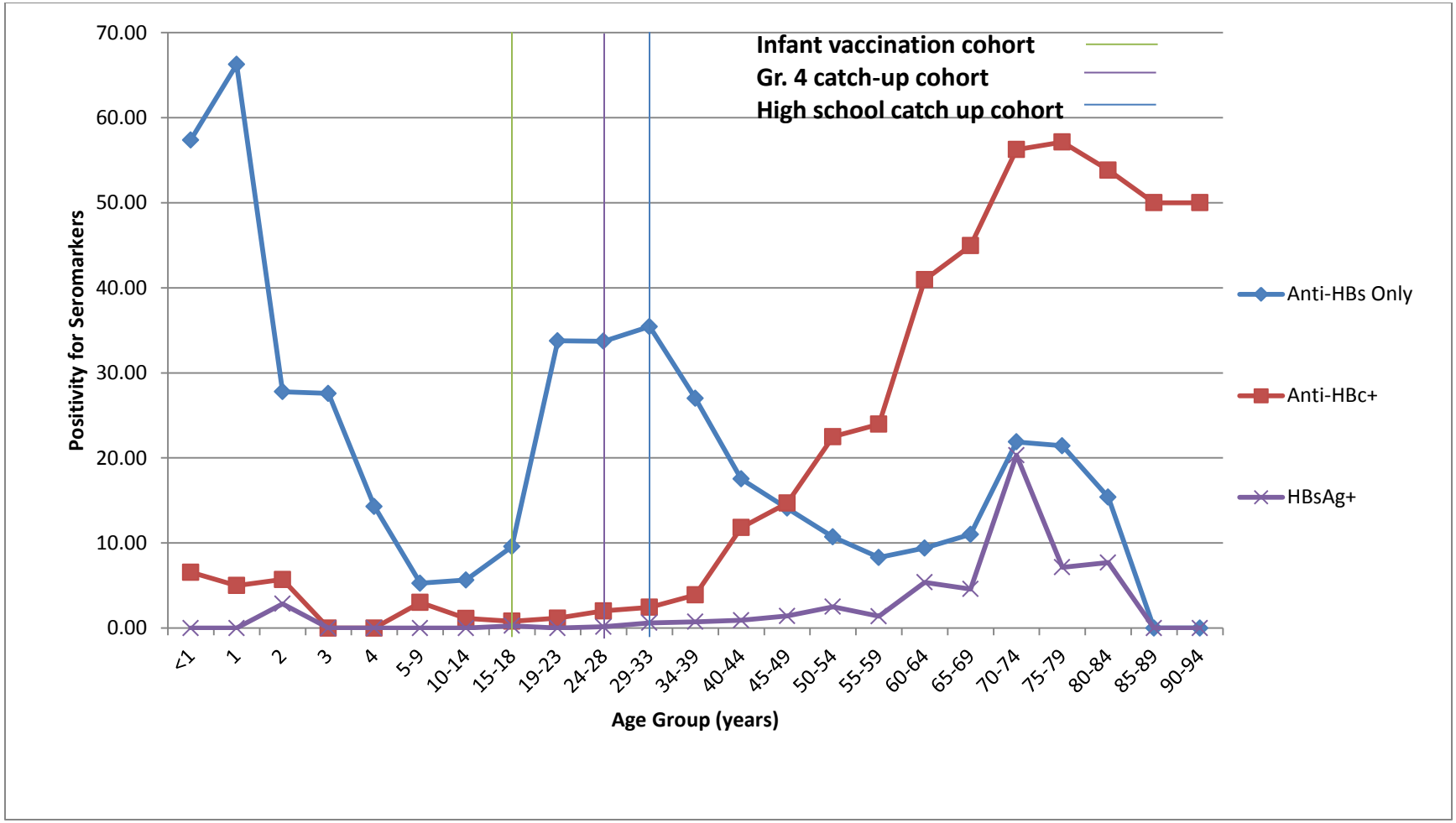


Figure 4: Prevalence of anti-HBs, anti-HBc, and HBsAg in the Nunavut sample population. A breakdown of the prevalence of anti-HBs, anti-HBc, and HBsAg within the noted age groups. Although prevalence of anti-HBs is very low within the infant vaccination cohort, HBV infection seromarkers are also very low, highlighting the potential effectiveness of Nunavut's vaccination program. The vertical bars in the centre depict the different vaccination cohorts: Infant vaccination from <1 to 18 years old (very left to green bar), grade 4 catch-up from 19 to 28 years old (green to purple bar), and high school catch-up from 29-33 years old (purple to blue bar).

Cohort	Anti-HBs only positivity	Anti-HBc+	HBsAg+
Total population	1119/4771 (23.5%)	448/4766 (9.40%)	56/4766 (1.17%)
Non-vaccinated	315/1965 (16.0%)	395/1963 (20.1%)	50/1963 (2.55%)
Vaccinated	804/2806 (28.7%)	53/2803 (1.89%)	6/2803 (0.21%)
- Infant vaccination	164/934 (17.6%)	19/933 (2.04%)	2/933 (0.21%)
- Grade 4 catch-up	466/1380 (33.8%)	22/1379 (1.60%)	1/1379 (0.07%)
- High school catch-up	174/492 (35.4%)	12/491 (2.44%)	3/491 (0.61%)

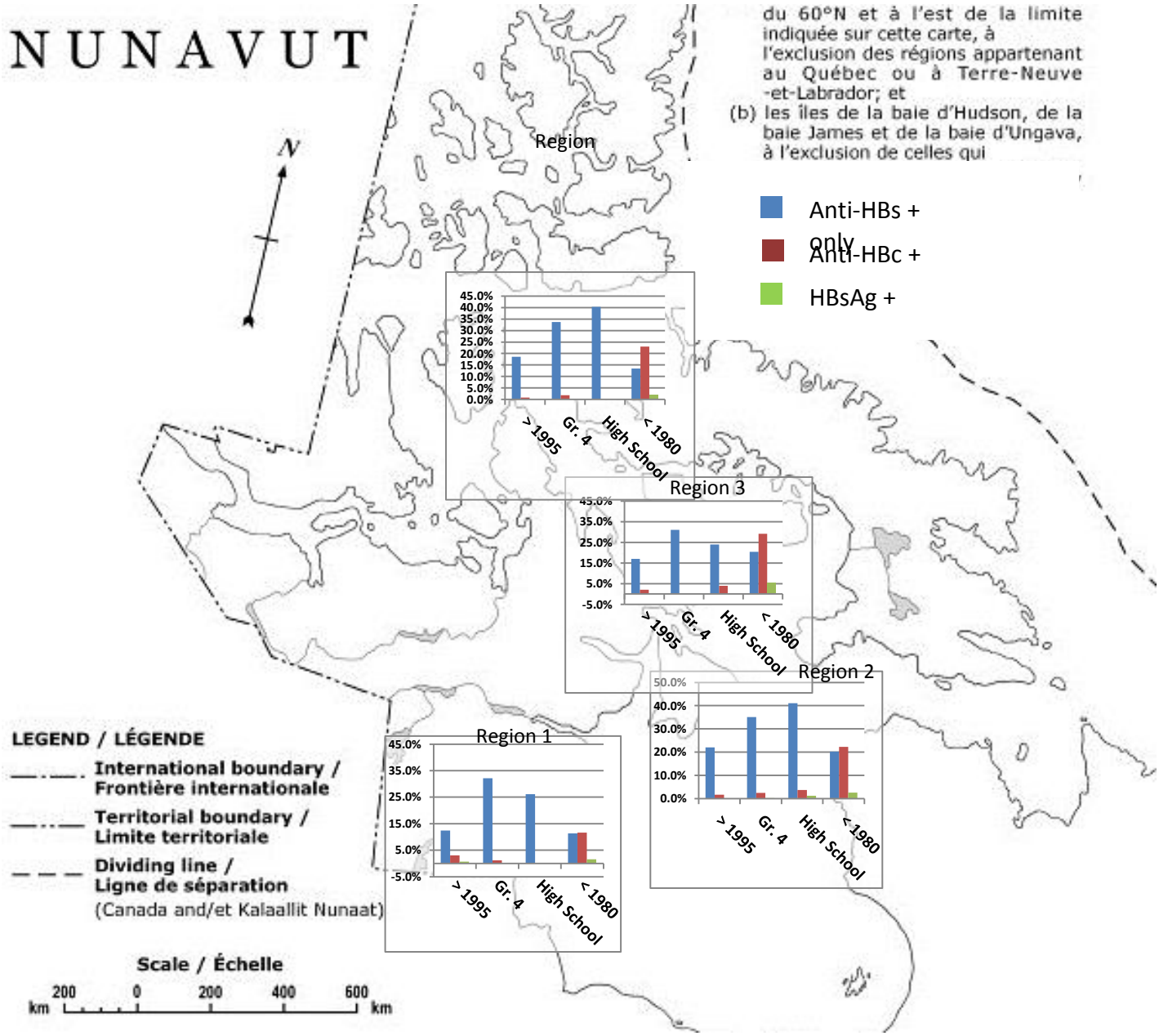
Table 6: HBV seromarker positivity by cohort. A breakdown of the prevalence of each HBV seromarker (anti-HBs, anti-HBc, and HBsAg) relative to each cohort, with large reductions in both HBV infection seromarkers from the non-vaccinated to vaccinated cohorts: anti-HBc – 20.1% to 1.89% ($p<0.001$) and HBsAg – 2.55% to 0.21% ($p<0.001$).

Samples tested were also separated into 4 geographical regions of Nunavut for regional analysis (Fig. 5). No large deviations from the overall trend for any HBV seromarkers compared to the entire sample population were observed in any region. Region 1 contained both potential breakthrough infections and the lowest anti-HBs levels within the infant vaccine cohort (Table 7). This may suggest the need to improve vaccination efforts within this region. Region 3 is the only region with over 2% HBsAg prevalence (2.2%, 12/551), however, all HBsAg positive samples were from individuals within the non-vaccinated cohort. Over time, the prevalence should continue to decrease as universal vaccination continues and the vaccinated cohort grows.

HBV DNA Analysis

All HBsAg positive samples were subject to amplification and sequence analysis in order to determine genotype, phylogenetic clustering with GenBank subgenotype sequencing, and mutational analysis. Forty-four of the fifty-six (78.6%) HBsAg positive samples were successfully amplified for either one or both targets; either the surface or precore coding regions.

Quantitative viral load testing was done on 42 of the 44 DNA positive samples (one NSQ/one undetermined). The samples had a mean viral load of 8.49×10^6 IU/ml, and a median of 489.8 IU/ml, ranging from 2.93 IU/ml to 2.17×10^8 IU/ml.



http://atlas.gc.ca

Figure 5: Nunavut map with regional prevalences of HBV seromarkers by cohort. Analysis of overall HBV seromarker prevalences dividing Nunavut into four geographical regions. No large deviations were observed for any regions compared to the overall prevalences within the sample population (Table 7).

BY REGION	Cohort	Total tested for Anti-HBs	Anti-HBs + ONLY	Anti-HBs ONLY %	Total tested for Anti-HBc	Total anti-HBc +	Anti-HBc %	Total tested for HBsAg	HBsAg +	HBsAg % (Based on tested anti-HBc samples)
1	> 1995	299	35	11.7%	296	9	3.0%	9	2	0.7%
	Gr. 4	429	138	32.2%	427	5	1.2%	5	0	0.0%
	High School	134	35	26.1%	134	0	0.0%	0	0	0.0%
	< 1980	645	73	11.3%	644	75	11.6%	75	10	1.6%
2	> 1995	373	81	21.7%	372	6	1.6%	6	0	0.0%
	Gr. 4	610	214	35.1%	610	15	2.5%	14	1	0.2%
	High School	246	101	41.1%	246	9	3.7%	9	3	1.2%
	< 1980	883	179	20.3%	882	196	22.2%	194	23	2.6%
3	> 1995	100	17	17.0%	100	2	2.0%	1	0	0.0%
	Gr. 4	171	53	31.0%	171	0	0.0%	0	0	0.0%
	High School	50	12	24.0%	50	2	4.0%	2	0	0.0%
	< 1980	230	47	20.4%	230	67	29.1%	66	12	5.2%
4	> 1995	124	23	18.5%	124	1	0.8%	1	0	0.0%
	Gr. 4	166	56	33.7%	166	3	1.8%	3	0	0.0%
	High School	62	25	40.3%	62	0	0.0%	0	0	0.0%
	< 1980	252	34	13.5%	252	58	23.0%	57	5	2.0%

Table 7: Analysis of HBV seromarkers by region. As mentioned previously, all four regions were similar for HBV seromarker prevalences and did not deviate from the overall Nunavut prevalences for the current study. The only region with >2% HBsAg positivity was region 3. However, all HBsAg positive samples from region 3 were within the non-vaccinated cohort (born before 1980), and therefore, with an increasing population within the vaccine cohort over time, the HBsAg prevalence will most likely decline.

Phylogenetic Analysis

A phylogenetic tree was constructed using surface or precore sequences to investigate sequence relationships. The surface phylogenetic tree was constructed utilizing 342 bp, ranging from nt 455 to 796 in the HBV genome (Fig. 6). The precore phylogenetic tree utilized 319 bp, which ranged from nt 1652 to 1960 in the genome (Fig. 7).

It is to note, a recent review has reclassified genotype B subgenotypes, specifically reducing the total number of subgenotypes of genotype B to B1 to B5 [55]. The Inuit population of the western circumpolar Arctic region were originally associated with genotype B6 infection, which has been redesignated as genotype B5. Discussion of genotype B5 within the current study is based on all past discussion and understanding of genotype B6.

Surface sequence is generally used for genotyping of HBV as precore sequence is not appropriate due to inherent recombination within the region and the lack of genotypic informative sites. Subgenotyping designation usually requires full genome sequence analysis [110]. All HBV/B study sequences clustered together within the same clade as reference genotype B5 sequences, which was expected as genotype B5 is the majority genotype in Nunavut. All HBV/A or HBV/D study sequences clustered with genotypes A1 and D4, respectively, although the surface region sequence analyzed was too small to adequately determine the subgenotype. It was determined 84% (36/43) of samples were HBV/B5, 14% (6/43) were HBV/A, and 2% (1/43) were HBV/D.

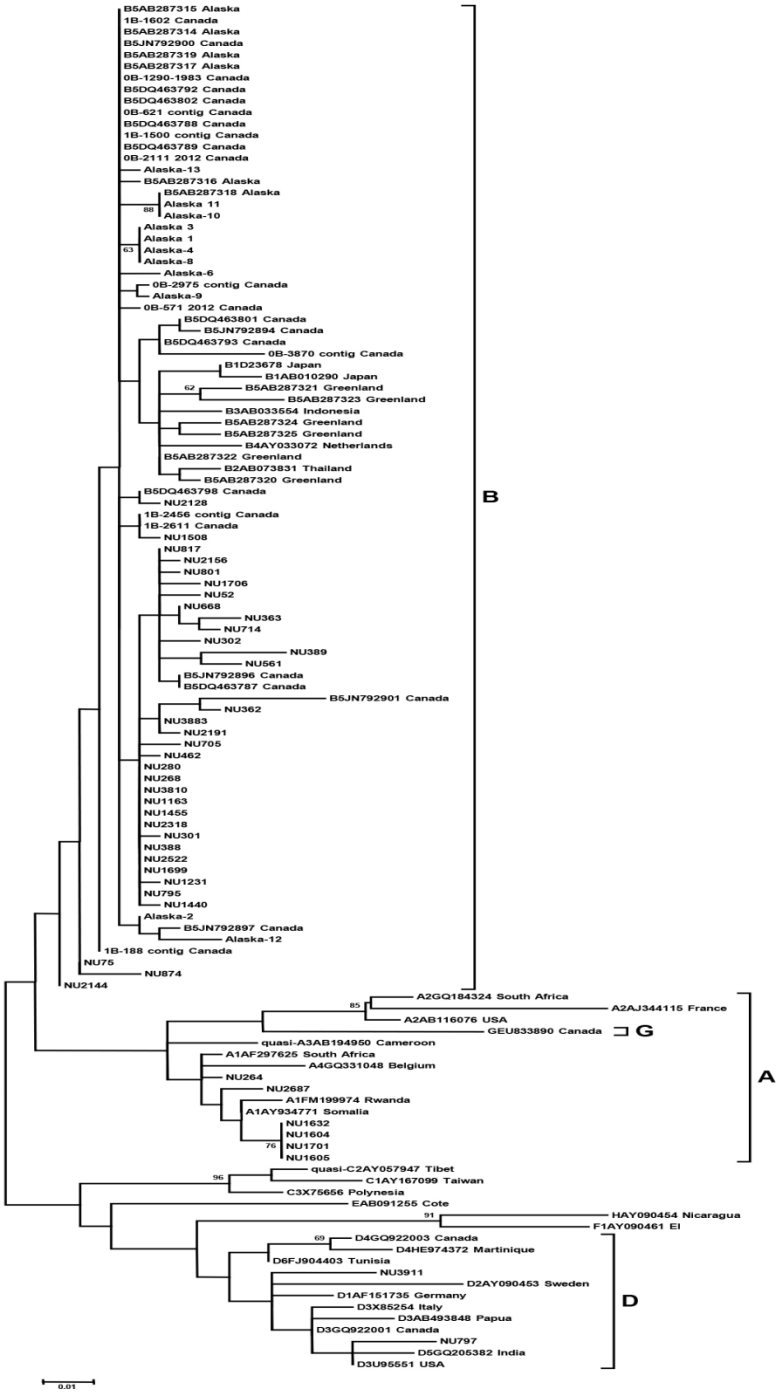
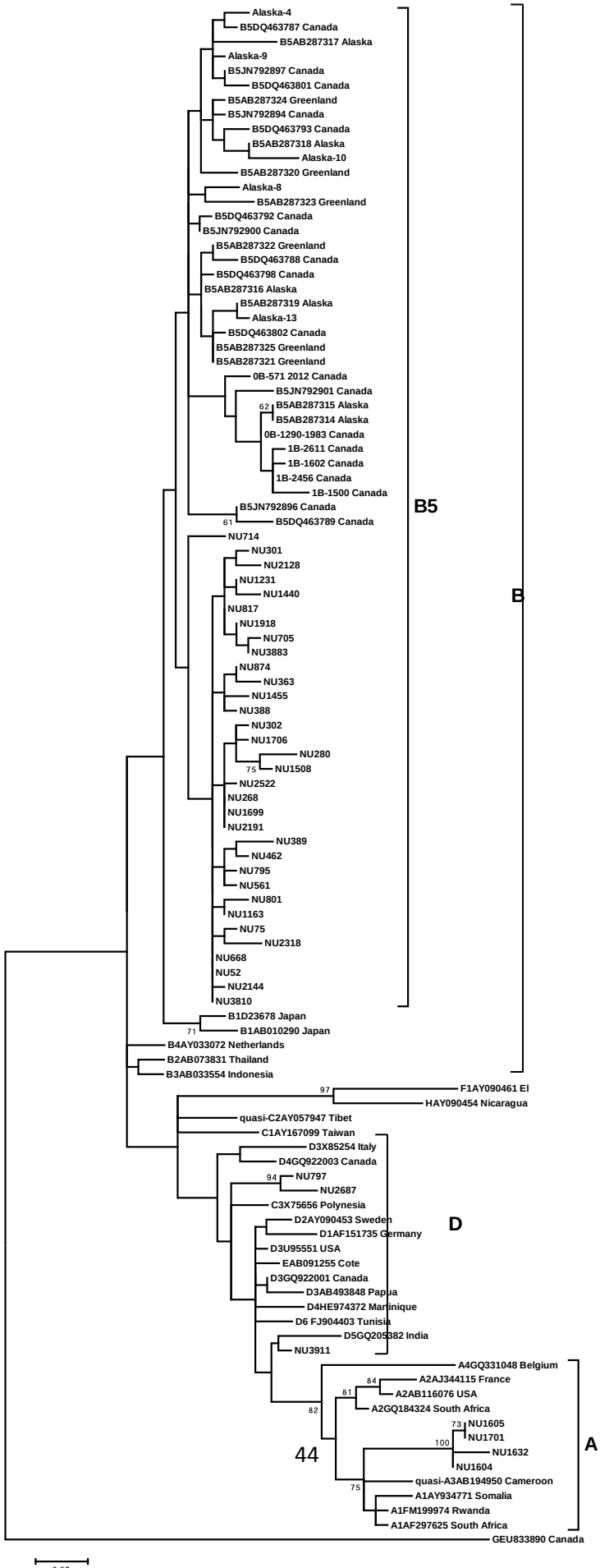


Figure 6: Phylogenetic analysis of the surface coding region sequence for Nunavut HBV samples (designated "NU#"). Forty-two sequences, consisting of 342 bp for the surface coding region, were aligned with GenBank sequences that included genotypes A, B, and D, which are shown as subgenotype/genotype followed by the GenBank accession number and country of origin, in order to determine sequence clustering and potential subgenotypes. Other internal B5 reference sequences used are designated as a country and ID number. Bootstrap confidence values are >=60% are shown. The ruler shows the branch length for a pairwise distance equal to 0.01.


Alaska-4
B5DQ463787 Canada
B5AB287317 Alaska
Alaska-9
B5JN792897 Canada
B5DQ463801 Canada
B5AB287324 Greenland
B5JN792894 Canada
B5DQ463793 Canada
B5AB287318 Alaska
Alaska-10
B5AB287320 Greenland
Alaska-8
B5AB287323 Greenland
B5DQ463792 Canada
B5JN792900 Canada
B5AB287322 Greenland
B5DQ463788 Canada
B5DQ463798 Canada
B5AB287316 Alaska
B5AB287319 Alaska
Alaska-13
B5DQ463802 Canada
B5AB287325 Greenland
B5AB287321 Greenland
0B-571 2012 Canada
B5JN792901 Canada
62
B5AB287315 Alaska
B5AB287314 Alaska
0B-1290-1983 Canada
1B-2611 Canada
1B-1602 Canada
1B-2456 Canada
1B-1500 Canada
B5JN792896 Canada
61
B5DQ463789 Canada
B5
NU714
NU301
NU2128
NU1231
NU1440
NU817
NU1918
NU705
NU3883
NU874
NU363
NU1455
NU388
NU302
NU1706
NU280
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NU1508
NU2522
NU268
NU1699
NU2191
NU389
NU462
NU795
NU561
NU801
NU1163
NU75
NU2318
NU668
NU52
NU2144
NU3810
B1D23678 Japan
71
B1AB010290 Japan
B4AY033072 Netherlands
B2AB073831 Thailand
B3AB033554 Indonesia
B
97
F1AY090461 El
HAY090454 Nicaragua
quasi-C2AY057947 Tibet
C1AY167099 Taiwan
D3X85254 Italy
D4GQ922003 Canada
94
NU797
NU2687
C3X75656 Polynesia
D2AY090453 Sweden
D1AF151735 Germany
D
D3U95551 USA
EAB091255 Cote
D3GQ922001 Canada
D3AB493848 Papua
D4HE974372 Martinique
D6 FJ904403 Tunisia
D5GQ205382 India
NU3911
A4GQ331048 Belgium
84
A2AJ344115 France
81
A2AB116076 USA
82
A2GQ184324 South Africa
73
NU1605
NU1701
100
NU1632
44
NU1604
quasi-A3AB194950 Cameroon
75
A1AY934771 Somalia
A1FM199974 Rwanda
A1AF297625 South Africa
A
GEU833890 Canada
0.02

Figure 7: Phylogenetic analysis of the precore coding region sequence for Nunavut HBV samples. Forty sequences, consisting of 319 bp for the precore coding region, were aligned with GenBank sequences that included genotypes A, B, and D shown as subgenotype/genotype followed by the GenBank accession number and country of origin. Other internal B5 reference sequences used are designated as a country and ID number. Bootstrap confidence values >= 60% are shown. The ruler shows the branch length for pairwise distance equal to 0.02.

Eight HBV/B samples were amplified for full length genomic sequencing, to compare with other reference HBV/B5 isolates with full genomic sequencing in order to determine if the subgenotype of the HBV/B of the current study were indeed genotype B5 and to compare current sequences to reference sequences by phylogenetic and distance-based methods.

The HBV/B samples from this study clustered within a monophyletic clade, most closely with other HBV/B5 reference strains from Baker Lake, Nunavut, with high bootstrap scores (Fig. 8), compared to other subgenotypes. Therefore, HBV/B samples within the study could be accurately described as genotype B5.

HBV Mutation Analysis

Specific mutations within the HBV genome have been shown to either increase or decrease the likelihood of end-stage liver disease within chronically infected patients. HBV/B5 has been shown to have a high likelihood of containing the precore mutation, G1896A, which may be a contributing factor in HBV/B5 causing an inactive, benign infection. All 36 HBV/B5 samples had this precore mutation and, in addition, no double mutation was observed within the basal core promoter (A1762T/G1764A) [76].

HCV Prevalence in Nunavut

In addition to testing for HBV seromarkers, samples were also tested for presence of antibodies to HCV. The sample population had a prevalence of 0.50% (24/4766) for anti-HCV

prevalence in Nunavut. All HCV infections affected individuals aged 24 to 69 years old (Fig. 9 and Table 8). HCV RNA analysis was not performed due to possible RNA degradation following variable length of time at 4^{o}C during storage prior to shipping and also during shipping, leading to unreliable testing and results.

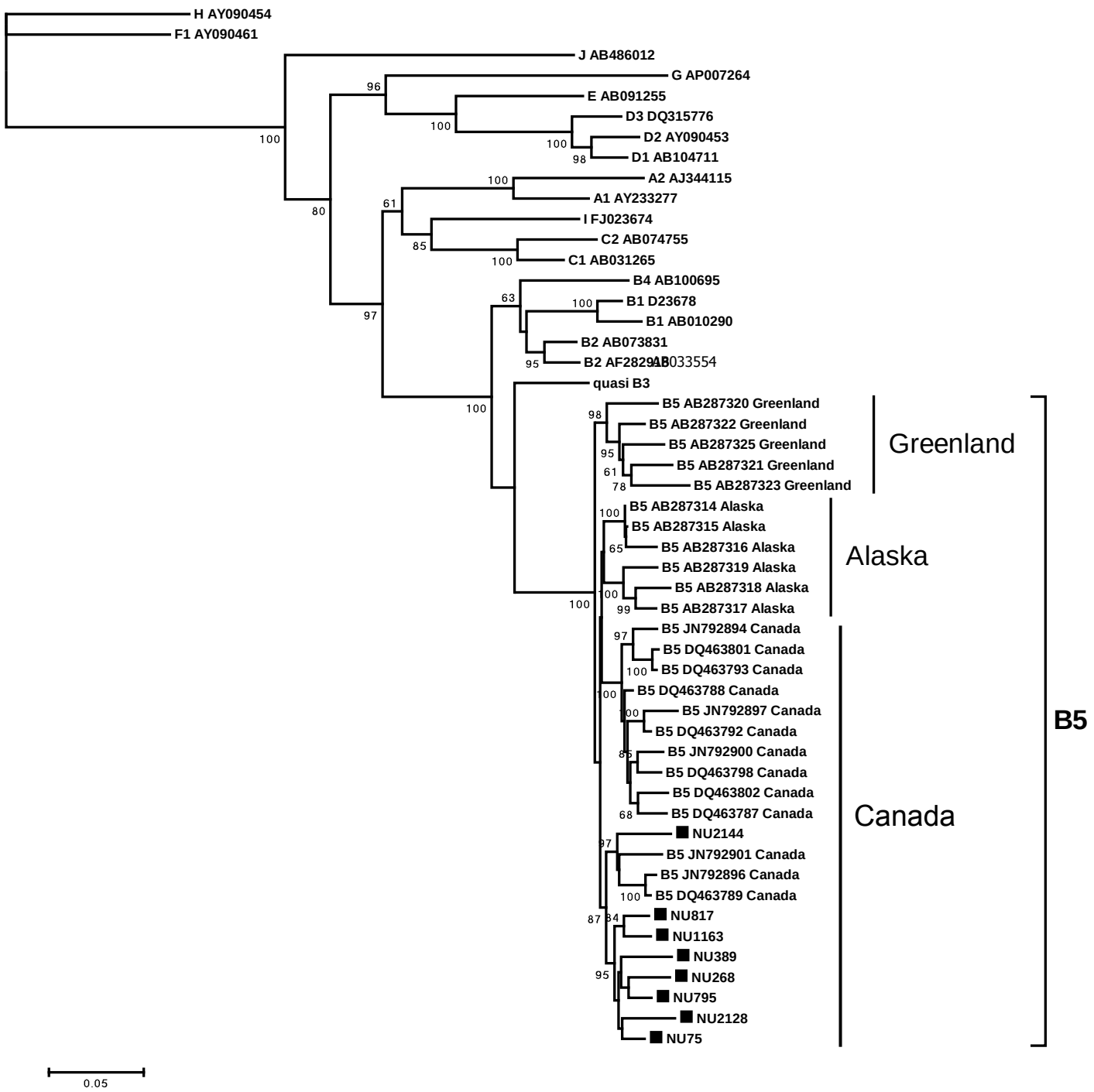

Figure 8: Phylogenetic analysis of the full genome sequences for eight Nunavut samples. GenBank reference sequences used are shown as subgenotype/genotype followed by the GenBank accession number and country of origin. Other internal B5 reference sequences used are designated as a country and ID number. Bootstrap confidence values >=60% are shown. The ruler shows the branch length for a

pairwise distance equal to 0.05. Phylogenetic clustering can be observed regionally for all genotype B5 samples used for analysis. Most Nunavut samples clustered together with a higher within group mean distance compared to other Canadian B5 samples, demonstrating higher variability and mutation rate of genotype B5 over time.

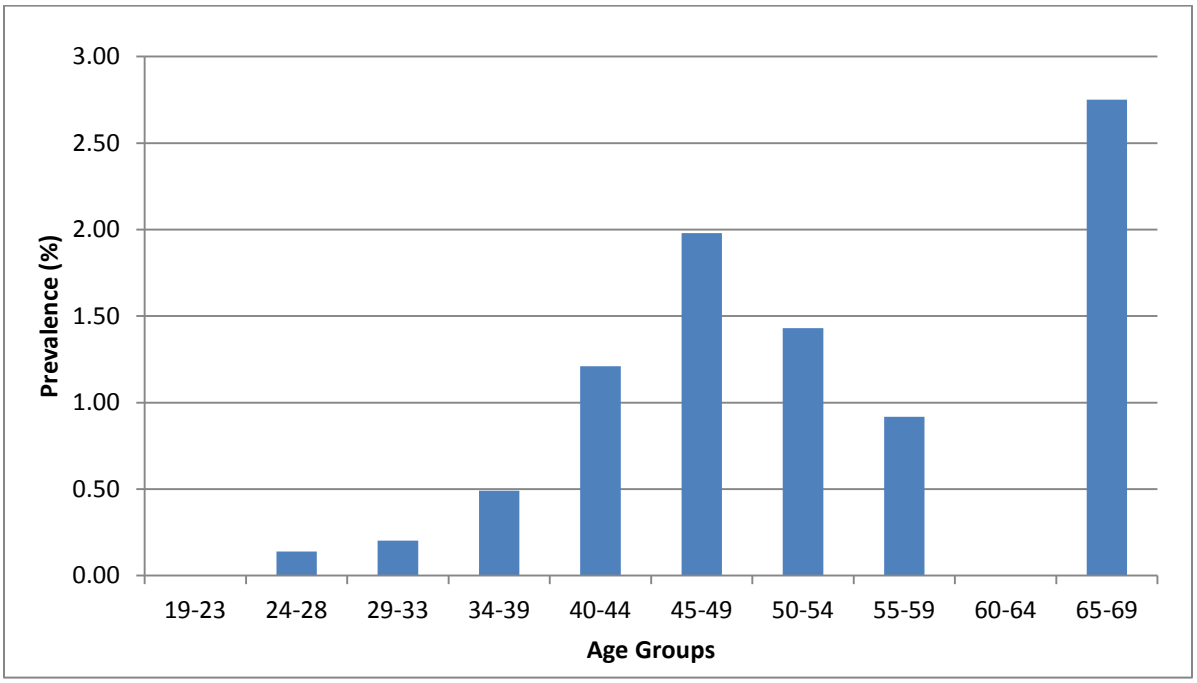

Figure 9: Prevalence of anti-HCV in Nunavut within respective age groups. The prevalence of HCV infection divided into approximately 5 year age groups. Overall prevalence of HCV in the sample population is 0.50%. Age groups not shown did not have anti-HCV positivity.

Discussion

The current study investigates the effectiveness of the universal vaccination program in Nunavut, including infant vaccination which continues presently, and two historic simultaneous catch-up vaccination programs. The impact of the vaccination program has decreased HBV prevalence to <2%, whereby Nunavut may now be considered non-endemic for HBV infection.

Within the sample population, 23.5% of tested individuals had protective levels of anti-HBs, without any other HBV seromarker present. Within the vaccination cohort, only 28.7% of

Age group	Total Samples	Anti-HCV+
19-23	686	0 (0.0%)
24-28	693	1 (0.14%)
29-33	491	1 (0.20%)
34-39	411	2 (0.49%)
40-44	330	4 (1.21%)
45-49	354	7 (1.98%)
50-54	280	4 (1.43%)
55-59	217	2 (0.92%)
60-64	149	0 (0.0%)
65-69	109	3 (2.75%)

Table 8: Prevalence of anti-HCV in Nunavut within respective age groups. Numerical anti-HCV positivity in Nunavut by age groups that were infected by HCV. Age groups not shown did not have anti-HCV positivity.

individuals had protective levels of anti-HBs. Protective anti-HBs levels of individuals who have recently received vaccination were 57.4%, 66.3% and 27.8% of individuals aged <1, 1, and 2, respectively (Fig. 4). This prevalence then drops to below 10% for those 5 to 18 years old. Persons aged 19 to 28 and 29 to 33, who were part of the grade 4 and high school catch-up programs, had an increase in protection, with 33.8% of the subpopulation having protective antibody levels. Individuals, who received vaccination later on in life, such as the grade 4 and high school catch-up cohorts, generally retain anti-HBs levels for a longer period and/or have

higher primary responses than those vaccinated in infancy, which explains the rise in protective antibody levels in these cohorts compared to the 5 to 18 age range [111-113].

The protective antibody levels were much lower than expected across all age groups within the vaccination cohort. The anti-HBs prevalence increase from individuals aged <1 to 1 year old could be due to the infants <1 years old not receiving all 3 doses of vaccination, leading to a potential decrease in individuals with protective antibody level. Incomplete vaccination has been highly associated with becoming an HBV carrier upon exposure to HBV, opposed to those fully vaccinated [114]. However, the rate of decay after primary and secondary doses in infancy has not been studied, and therefore the lowered protective anti-HBs level in infants <1 years old may or may not be attributable to lack of full vaccination. The most unexpected result were with those aged 5 to 18, where less than 10% (5.26% ages 5 to 9; 5.65% ages 10 to 14; 9.57% ages 15-18) of the subpopulation had protective antibody levels.

The current study was limited by not having vaccination records and background information for its participants, and could not reliably investigate why the lower unexpected values were discovered. However, there are reasonable explanations why these levels were observed, which include antibody decay, program delivery and infrastructure, and maintaining proper cold-chain for the vaccination.

Infrastructure failure, within the vaccination program itself, may have led to a reduction in anti-HBs seroprevalence. Nunavut covers a large area of northern Canada with isolated communities, and is located within the Arctic, which poses unique challenges for delivery of timely health care. Given the individuals with the poorest antibody levels are aged closest to the beginning of the vaccination program's implementation, they may not have received a full

vaccine regimen as there may not have been a standard operating procedure throughout Nunavut at the start of the program. Health care workers who have worked within this region also allude that health care is not always readily available to the entire population, due to the unique geographic challenges in Nunavut, which may lead to difficulties in administering health care and subsequent vaccination doses [115].

In addition, the transportation of vaccine early in the program to all the communities may have resulted in the vaccines freezing. Proper cold-chain is required for the HBV vaccine to remain effective, and freezing is known to lead to vaccine inactivation [72].

Antibody decay could also explain the diminished anti-HBs levels within these individuals. Previous studies have shown that rate of antibody decay is independent of vaccine type or brand, protocol, or gender [116-117]. However, this may not be the case when comparing between endemic, high-risk and low-risk, non-endemic regions. Long-term follow-up studies are now being completed investigating antibody levels after initial infant vaccination in endemic regions, like Thailand and high-risk regions of Alaska [4,71]. However, these regions generally have slower antibody decay and more persistent levels over time. Both regions had almost perfect vaccination coverage with over 95% of infants immunized, and after 20 years, over 50% of the tested population still retained protective antibody levels (Fig. 10).

Low-risk groups in Alaska, such as children born into families having no chronic HBV infection, as well as other low-risk countries (Italy, Iran, and USA (Hawaii and low-risk Alaska)), had more rapid antibody decay (Fig. 10) [117-120]. By the time individuals in these regions reach 10 years old, only 15-20% of the population retain protective anti-HBs levels.

Nunavut is considered a high-risk, endemic region that had a prevalence of over 3% before this study, and therefore, it was believed that the antibody decay rate would have trended like other

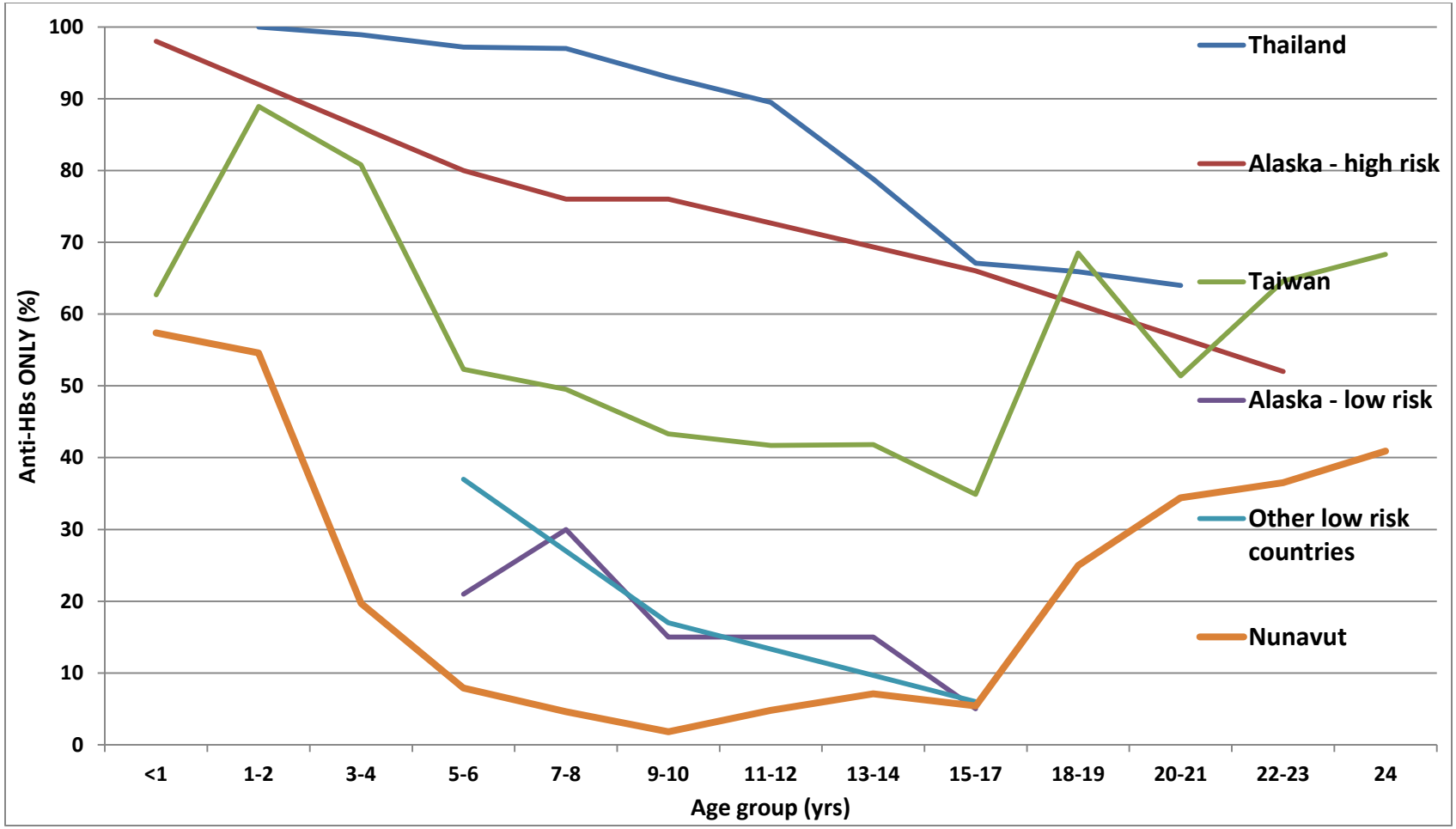

Figure 10: Comparison of Nunavut anti-HBs prevalence with other high and low-risk regions for HBV infection. High-risk regions for HBV infection, such as Thailand, high-risk Alaska, and Taiwan, generally have a much slower anti-HBs decay, potentially due to natural immune boosting from environmental exposure to the virus. Low-risk regions (Italy, Iran, and USA (low-risk Alaska and Hawaii)) start with high initial anti-HBs levels, but decay at a much more rapid pace, even though these regions immunize at birth. Although not all age groups are shown, low-risk populations were also vaccinated at birth with follow-up starting 5-6 years post-vaccination. Nunavut was expected to have a much slower anti-HBs decay, but as shown, the anti-HBs levels are much lower than anticipated.

high-risk, endemic regions. However, this was not observed, as Nunavut follows more closely with low-risk trends. The vast geography of Nunavut and isolated communities may explain why antibody levels decay much more rapidly than expected as well as having a historical prevalence on the low-end of the endemic spectrum (3% vs. 6% in Alaska or 9.8% in China) [9,87]. In high-risk regions, antibody levels may remain high in the population due to natural boosting, where constant re-exposure to HBV would lead to persistent antibody production within the host [99].

The WHO recommendation that at least 80% of the infant population be appropriately vaccinated appears to not have been met in the current study, at least based on the prevalence of protective anti-HBs in the infant vaccine cohort, and in comparison to other HBV endemic populations. As the correlation between infant HBV vaccination and antibody decay over time is not completely understood and is highly dependent on a number of variables (community endemicity, host effects, etc.), it is premature to conclude that recommended vaccination targets were not met, and require further study, as will be discussed shortly.

A host factor may also explain low antibody levels. Nunavut is heavily populated by an Inuit population, and no follow-up post-HBV vaccination has been done. Therefore, the indigenous population may not retain a humoral response against HBV or mount an effective primary response very well compared to other populations, globally. It has been shown that nucleotide variation in key cytokine/cytokine receptor and toll-like receptor genes are associated with responder and non-responder status to HBV vaccination-induced protective humoral immunity [121]. Studies of Australian Aboriginal populations have also shown reduced levels of humoral protection even though full HBV vaccination was given in infancy [122]. It was suggested the decreased immune response is due to potential genetic variation in this population [123-124].

Although the anti-HBs levels in this study are lower than anticipated, the universal vaccination program seems to have had a positive effect and has been successful in reducing the prevalence of HBV infection in Nunavut. Comparing the unvaccinated and vaccinated cohorts, a prevalence of 20.1% and 2.55% to 1.89% and 0.21% for anti-HBc and HBsAg positivity, respectively, clearly shows a significant reduction in HBV exposure and chronic infection due to vaccination ($p<0.001$). Anti-HBc exposure may truly be lower than reported within the infant vaccination cohort, as anti-HBc positivity in the infant's (up to 2 years of age) blood may be due to long term persistence of maternal antibodies [125]. Indeed, despite the observation of exposure to HBV (anti-HBc positivity) throughout the infant vaccine cohort, no or very low levels of HBsAg positivity developed, indicating a transient exposure, likely resolved due to vaccine-based immunity. The decrease in HBV infection seromarkers suggests, even though overall anti-HBs prevalence is low in the vaccine cohort, universal vaccination is still successful, and perhaps reasoning other than lack of sufficient vaccination coverage may explain the low anti-HBs prevalence in the population.

However, the universal infant vaccination may not have complete coverage as two specimens within the infant vaccination cohort were positive for HBsAg and HBV DNA. There were no vaccine escape mutations within these isolates' HBsAg coding region, and each had an undetectable anti-HBs level, which suggests these individuals may not have received full vaccination. One of these isolates may be due to immigration, and will be discussed later. Six other individuals within the catch-up vaccination cohort were also positive for HBsAg. These individuals may have been infected prior to receiving a vaccination, which would not resolve progression of a chronic infection.

The western circumpolar region has been regarded as an area with high levels of hepatitis for many years since initial seroepidemiological surveys were conducted. In addition, this region is heavily populated by indigenous populations, who have a 10 to 20 times higher risk of HBsAg-positive carrier rate [126]. As mentioned previously, one of the first major surveys that investigated the Canadian Arctic region for HBV prevalence was conducted in the mid-1980s which observed an overall prevalence of approximately 3% [8]. Studies specifically investigating Inuit communities within the Baker Lake region of Nunavut conferred with these results at the time with a similar HBV prevalence of 4% [127-128]. Baikie et al. investigated inhabitants of Northern Labrador, and discovered an overall HBsAg prevalence of 3.2%, with Inuit-specific HBsAg carrier rate to be significantly higher (6.9%) [69]. Currently, approximately three decades since these studies, a large reduction of HBV prevalence in the general population of Nunavut is observed. The current study is the first documented indication of a significant reduction in HBV prevalence (1.17%), with the vaccination age cohort prevalence reduced to 0.21%. The HBV prevalence is expected to continue to decline for the next several years as the vaccination population increases relative to the general population over time.

Overall HCV prevalence in the sample population was also relatively low at 0.5% (24/4766), with only individuals aged 24 to 69 being afflicted (Fig. 9). HCV is highly associated with the ‘baby boomer’ generation, individuals born between 1945 and 1965, and approximately aged 49 to 69 at the time of this study. Like HBV, HCV is transmitted by blood and reasoning for the high incidence within this subpopulation is not completely understood. But it is believed contact with contaminated blood and blood products, which includes blood transfusions, was a primary way of HCV spreading through the population [129]. Universal blood supplies were not screened for bloodborne pathogens until 1992, when adequate precautions were adopted.

Intravenous drug use is most likely another contributor to HCV transmission. No HBV/HCV co-infections were observed during the present study.

HCV prevalence in Canada is estimated at 0.8% [130]. The current study investigating HCV prevalence in Nunavut indicates a lower prevalence relative to the national estimate within the study population at 0.5%. This may be unexpected as North American indigenous populations tend to have a higher risk and carrier rate for anti-HCV, including a study of two central First Nations communities in Canada which found an HCV seropositivity rate of 2.2% [131]. Canadian data also show a high HCV incidence rate of 18.9 per 100,000 for Canadian Aboriginals relative to a rate of 2.8 per 100,000 for non-Aboriginal Canadians [132].

In northern circumpolar Canada, region-specific HCV seroprevalence ranged from 1% to 15% [133]. However, other circumpolar regions outside of Canada had much lower prevalence rates. In Greenland, seropositivty for HCV was 0% to 1.5% and Alaska Native individuals had a seroprevalence of 0.8% [134-136].

Interestingly, it seems HCV infection outcome may be dependent on ethnicity and origin. Greenland and Nunavut are primarily populated by individuals of Inuit descent, and both regions have a relatively low HCV prevalence rate. In addition, a survey of a Manitoban First Nations reserve community documented an anti-HCV prevalence of 2.2%, but an HCV-RNA positivity rate of less than 0.1%. [131]. Recent studies suggest unique host immunity may enhance spontaneous clearance of HCV in certain Aboriginal populations [137-138]. One study compared Caucasian peripheral blood mononuclear cell (PBMC) interleukin-10 (IL-10) production to First Nations PBMC IL-10 production [137]. In response to HCV core protein, Caucasian PBMCs produced significantly higher IL-10, and First Nations PBMCs produced relatively low levels of

IL-10. IL-10 is an anti-inflammatory factor, which HCV can induce as an immune escape mechanism. The low level of IL-10 production by First Nations subjects suggests reduced susceptibility to HCV and enhanced viral clearance, leading to lowered risk to develop chronic infection, which may explain the low HCV prevalence in Nunavut relative to the national prevalence [137]. However, common risk factors for HCV transmission may also not be as prevalent in Nunavut as in the rest of the country, which may account for these findings.

Other hepatitis viruses also affect the western circumpolar region. Although no recent follow-up has been done regarding hepatitis A virus (HAV) prevalence, past studies have documented prevalence rates of total hepatitis A antibody of >50% in Alaska and 82% in two anonymous Inuit communities in Canada [133,139]. These high prevalence rates are expected to have decreased as vaccination against HAV is available and offered as part of the childhood vaccination schedule. Little evidence is available for hepatitis D virus (HDV), which requires co-infection with HBV, epidemiology within the Arctic region. It was reported 40% of HBV infected persons in Greenland also carried HDV. However, with the decrease in HBV due to vaccination, HDV prevalence was also expected to drop, and now only 1.1% of persons in Greenland have serological signs of HDV exposure [135].

A prevalence of 3% for hepatitis E virus (HEV) amongst Canadian Inuit within the Arctic region was reported [140]. Consumption of deer meat has been reported to transmit HEV, via fecal-oral route. Deer and caribou are closely related and caribou meat is a staple of the Canadian Inuit diet, which may explain the high HEV prevalence amongst Canadian Inuit.

Phylogenetic analysis revealed the majority genotype in Nunavut was still HBV/B5, based on full genome sequencing, which amounted to 84% of the DNA positive samples in this

study, while HBV/A and HBV/D accounted for 14% and 2% of the DNA study sample, respectively. Like the majority of HBV/B5 isolates in other studies, all B5 samples in this study contained the precore G1896A mutation, which abolishes HBeAg production, and has been shown to lower the risk of cirrhosis and HCC [76]. The genotype B5 isolates of this study also do not have the basal core promoter double mutation (A1762T/G1764A), which has been associated with an increased risk of end-stage liver disease.

With closer analysis of tree topology, which includes all HBV sequences from this study along with GenBank reference sequences, HBV/B5 samples from this study clustered together based on surface sequence, and apart from the reference HBV/B5 sequences, but with a low bootstrap score (<60%)**.** This may be due to analyzing a small fragment of the surface coding region. Precore phylogenetic analysis revealed larger variability within the genotype B5 sequences. This may be partly explained by HBV/B5 isolates' ability to have increased variability in the precore/core region relative to other genotypes. Mutational analysis of western circumpolar genotypes (HBV/B5, HBV/D, and HBV/F) has shown HBV/B5 has higher variability and increased mutation rate compared to HBV/D and HBV/F [141].

Furthermore, analysis of HBV/B5 full genome sequences increasingly support the notion of genotype B5 having persistently increasing variability as a genotype population compared to other genotypes. Eight isolates that were identified as HBV/B by phylogenetic analysis utilizing surface coding sequence were amplified for full genome sequencing. Much like the clustering observed with the surface coding phylogenetic tree, the full genome tree was very similar, whereby the HBV/B samples from the current study cluster together into a single monophyletic clade, apart from previous genotype B5 GenBank sequences, with very high bootstrap values

(>80%). Because the full genome HBV/B sequences clustered with reference genotype B5 sequences, the HBV/B samples within the study can be confidently assessed as genotype B5.

Genotype B5 reference sequences used for full genome phylogenetic analysis included DQ463787, DQ463788, DQ463789, DQ463792, DQ463793, DQ463798, DQ463801, DQ463802, JN792894, JN792896, JN792897, JN792900, JN792901, AB287314, AB287315, AB287316, AB287317, AB287318, AB287319, AB287320, AB287321, AB287322, AB287323, and AB287325 from GenBank. DQ and JN sequences originated from a long-term longitudinal study analyzing changes in genotype B5 full genome sequence over 25 years [142]. DQ sequences were from samples originally isolated in either 1979 or 2004, and JN samples were from 2009. AB sequences were from a recent study analyzing full length genomic sequences in the western Arctic region [141].

Based on phylogenetic clustering and tree topology, it is clear genotype B5 has gained regional diversity over time. Samples from past studies originating from Greenland, Alaska, Northern Canada and the current study, all cluster into separate clades (Fig. 8). One sample from the current study did not cluster with the other seven full genome sequences from the current study. Upon sequence analysis, this sample had more substitutions than the other Nunavut B5 sequences, resulting in increased nucleotide distance, and thus led to clustering apart from these samples.

Group mean genetic distances were also calculated among full genome sequences in order to determine sequence divergence between the Nunavut genotype B5 samples compared to reference Canadian, Alaskan, and Greenlandic B5 samples, and also the group mean distance within each set of sequences.

As suspected, the most recent Nunavut genotype B5 samples of the current study have the highest within group mean distance (2.2%), slightly higher than the selection of full genome Canadian B5 sequences analyzed (2.0%) which were comprised of samples taken in 1979, 2004, or 2009 from 8 different individuals observed previously. The increased diversity within the recent Nunavut B5 samples compared to older Canadian B5 sequence further supports the characteristics of increased variability and mutation frequency of genotype B5 [141]. However, the older mean age (64.8 years) of individuals from which full length HBV sequences were obtained for this study and their varied geographical location may also contribute to the increased nucleotide diversity observed among this group compared to previous Canadian B5 sequences. Within group mean distances including Alaskan and Greenlandic B5 range from 1.8% to 2.5%.

Between group mean distances were also calculated. Nunavut B5 samples from the current study have a distance of 2.6% and 3.1% to Alaskan and Greenlandic B5 samples, respectively. These results were not surprising as previous reports have shown similar genetic distances between the Alaskan and Greenlandic sequences to previous Canadian sequences [141].

Although no clinical background information is known about the patients infected with HBV/B5 in this study, Canadian Inuit infected with HBV/B5 are known to generally have consistently normal liver biochemistry and low viral load levels, which is characteristic of an inactive carrier state [76-77,141]. Patients in a permanent inactive carrier state will generally not have further progression of liver disease.

Eighty-one percent (29/36) of HBV/B5 isolates from this study had a viral load of less than 2000 IU/ml. HBeAg was not tested for in this study, but due to the G1896A mutation, it can be assumed all HBV/B5 patients had seroconverted to anti-HBe. It has been shown HBV isolates from patients who have seroconverted to anti-HBe have higher genetic variability than non-seroconverters, and thus have more genomic variation than virus from HBeAg-positive persons [143-144]. Increased mutation rates may be attributed to selective pressures during the immune active phase which leads to lowered HBV DNA levels and increased HBV mutation in order to allow for immune escape [143,145].

HBV/B5 is very unique as it rarely causes cirrhosis or HCC, hallmark end-stage liver diseases which HBV is highly associated with as being the causative agent. The high amount of variability compounded with the lack of disease progression suggests that these characteristics of genotype B5 are due to potential host-pathogen balance [141]. HBV/B5 has only been associated with individuals self-identifying as Inuit within the circumpolar region, and preliminary evidence from geo-spatial phylogenetic analysis of full length genome sequences from Alaska, Canada, and Greenland suggests the evolution or "migration" of the virus in an eastward manner, from Asia to North America and Greenland (data not shown). This would support HBV/B1 being closely associated to HBV/B5, phylogenetically and with clinical outcomes, and suggests HBV/B1and HBV/B5 share a common ancestor predating estimated migration waves associated with present day Inuit [146]. The long-term relationship between host and pathogen may have led to the evolution of a 'pathogen attenuation' towards the host [147].

Both HBV/B5 and HBV/D were expected in the sample population as previous studies have shown [78]. However, the HBV/A strains detected, which were most similar to subgenotype A1, have not been previously observed in Nunavut. HBV/A1 is highly prevalent in

sub-Saharan Africa, and no literature has described this genotype in the western circumpolar region. However, HBV/A2 has been described in Kivalliq and throughout Canada [148]. The most likely explanation for the potential presence of HBV/A1 in Nunavut is immigration. But without any background information of the participants in this study, this is speculation. HBV/D is most likely associated with First Nations Dene individuals who immigrated to Nunavut from the Northwest Territories.

The current study has notable limitations. As mentioned throughout the article, having no background or historical information about the participants in this study limited the potential full understanding about HBV currently in Nunavut. Without vaccination records, it is unsure why protective anti-HBs levels are so low compared to other endemic regions that have had a very high success with their vaccination programs. Discussion regarding protective anti-HBs as the sole seromarker was all based on assumption of individuals born within the vaccination age cohort actually receiving full vaccination. However, this may not truly be the case. Vaccination records will need to be investigated in order to determine if lack of immunization early on in the universal vaccination program was in fact an issue. Other endemic regions have over 90% coverage within the first year of infants' lives in the respective regions, but Nunavut's <1 year old population within this sample only has a coverage of 57.4%.

Utilizing only three identifiers, as described within the ethical regulations to maintain patient anonymity, was also a limitation. By using date of birth, gender, and community of the sending health care centre, it is unlikely duplication of samples from the same patient occurred, but, duplication may have occurred if a patient had medical attention in a secondary community. Exclusion of individuals born the same day, gender, and in the same community may have also occurred. The decision to exclude samples with exact matching identifiers as an already received

sample far outweigh the sample bias if all samples received were included in the study, as multiple samples with identical identifiers, seemingly from the same patient, were present in many sample shipments.

Having a population based solely on individuals who visited health clinics over the year during sample collection may have also caused sample bias, opposed to having a truly random study. The study is heavily weighted in female to male participants (64.2% to 35.8%), which is understandable as the female population generally visits health care facilities more often. And although the preventative vaccine study should not be affected by an imbalance of female to male representatives, as all vaccine recipients are equally protected, having a more representative sample population of Nunavut would have been ideal [117]. The lack of participants aged 0 to 19 years old is also most likely due to how samples were collected, as younger individuals will not visit health clinics as often to have blood drawn as an older cohort.

Lastly, it is unclear what the exact date was for the inception of the universal vaccination program. Current healthcare providers and administrative officials in Nunavut were unable to provide a specific date in 1995, but EpiNorth reports do specify that the infant and grade 4 catch-up program started that year [149-150]. The current study assumes the program began at the start of the calendar year. If this was not the case, individuals born in 1995 before the start date would not have received a vaccination. In addition, both catch-up vaccination programs mentioned in the study are assumed to capture specific age cohorts. Since prevalence data is based on age, and age during a given school year can vary depending on date of birth, some individuals may be included in the vaccination age cohort when, truly, they were not within the specified grade levels receiving vaccination.

Conclusion

Nunavut was regarded as a high risk, endemic region for HBV infection prior to the implementation of a universal vaccination program. Presently, after two decades of universal vaccination, Nunavut now has an estimated overall prevalence of 1.17% for chronic HBV infection, and a vaccination age cohort prevalence of 0.18%. An approximate 10-fold decrease for both anti-HBc and HBsAg seromarkers was observed in the current study between non-vaccinated and vaccinated cohorts, suggesting a very successful and effective vaccination campaign alleviating HBV from the region. HBV/B5 continues to be the genotype observed affecting chronically infected patients in Nunavut.

Although lower than expected vaccine-based immunity will require follow-up to understand the causes and consequences, continued immunization should lead to Nunavut being considered a low-risk region for HBV infection.

Future Steps

The low prevalence of protective antibody levels in the Nunavut vaccination population may call into question whether a 4^{th} dose of vaccination is required in Nunavut, so individuals who live in this region may retain a more persistent humoral response and antibody levels to HBV. From 1984 to 1992, Taiwan utilized a 4 dose vaccination schedule (0, 1, 2, and 12 months of age) for all routine universal infant vaccination before switching to the universal 3 dose vaccination schedule [90]. Which of the dosing schedules is better is arguable. Taiwan, utilizing

both 3 and 4 dose vaccine schedules, had an overall prevalence of 50.5% for protective anti-HBs levels within the tested vaccination age cohort after a 20-year follow-up [90]. Alaska's vaccine age cohort, with just the universal 3 dose vaccination schedule, had a prevalence of 60% for protective anti-HBs levels [71].

The first step in determining if individuals in Nunavut are sufficiently protected by the 3 dose vaccination schedule is a booster study. Vaccinated individuals, whose vaccination records would be verified, would be administered a booster dose for follow-up, and anamnestic responses would be recorded. Generally, anti-HBs levels will increase over the protective threshold (>10 mIU/ml) by day 14, and by day 60 at the latest [18]. If a large portion of individuals do not respond to a booster dose, a 4^{th} HBV vaccination dose will be recommended as part of the universal vaccination schedule and booster doses for juveniles and teenagers would be recommend as well.

Other follow-up in the region includes verifying vaccination records to determine sufficient vaccination coverage and investigating potential host factors leading to quicker than expected antibody decay if vaccination records reveal that there is adequate coverage in Nunavut.

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