The Detection of Antibodies against Human Betaretrovirus Surface Protein in Patients with Primary Biliary Cirrhosis

By

Mark Kneteman

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Department of Medicine

University of Alberta

Abstract

Introduction: Primary biliary cirrhosis is an autoimmune liver disease in which cellular and humoral immune responses towards cholangiocytes cause progressive intrahepatic bile duct loss. Cholestasis, liver fibrosis, and eventually cirrhosis result, with liver transplantation or death occurring in end stage disease. The etiology of the disease is unknown, but is thought to involve a permissive genetic predisposition and an environmental insult. Plausible environmental insults include xenobiotic exposure, and bacterial or viral infections.

Previous work in our group suggests infection by human betaretrovirus, a virus with close similarity to mouse mammary tumor virus, may be associated with PBC. We are developing an ELISA based assay with the goal of establishing whether patients with primary biliary cirrhosis produce antibodies to human betaretrovirus. We also seek to produce a diagnostic indirect ELISA for detecting prevalence of human betaretrovirus in patients with primary biliary cirrhosis and the general population.

Methods: Human betaretrovirus Surface (gp52 Su) protein was expressed as a recombinant antigen from both *E. coli* and human cells. Affinity tags co-expressed with the antigens, GST for *E.coli* antigens and polyhistidine residues for mammalian antigens, were utilized to purify expressed antigen. Western blot was used to confirm successful antigen expression.

ELISAs were developed and optimized for both *E. coli* and mammalian expressed antigens. These ELISAs were utilized to probe healthy control and PBC patient serum samples for antibodies against human betaretrovirus surface protein. Western blot was used to validate the findings from the mammalian antigen ELISA.

Results: Three of four *E. coli* Human betaretrovirus Surface antigens were expressed as well as the mammalian antigen. While preliminary results from the *E. coli* antigen ELISA were promising, larger studies failed to reach significance. A preliminary ELISA utilizing mammalian expressed protein, the human betaretrovirus gp52 Su ELISA version 1, produced significant results with 20% of PBC patients exhibiting seroreactivity compared to 4.5% of healthy controls. A second version of the ELISA produced by InBios from the protocol of the first ELISA also produced significant results, and was a larger study with approximately 200 samples from healthy controls, PBC, primary sclerosing cholangitis and non-biliary liver disease patients. In this study, 20% of PBC patients had seroreactivity versus 3% of controls. In contrast, the HBRV gp52 Su ELISA version 3 detected reactivity in 4% of PBC patients versus 2% of controls. Western blot studies of selected samples (n=42) only demonstrated reactivity in one control and one PBC patient. Of these patients, none were positive samples by the HBRV gp52 Su ELISA version 3. Therefore, Western blot results did not validate the ELISA results.

Discussion: The results of the first two versions of the HBRV gp52 Su ELISA suggest that a portion of PBC patients produce anti-HBRV humoral immunity. However, these results must be validated by a secondary immunoassay that preserves conformational epitopes, and must be replicated with further ELISA studies. Preliminary Western blot results suggest some PBC patients and controls produce anti-HBRV gp52 Su antibodies to linear epitopes of HBRV Su. The sensitivity of the HBRV Su ELISA was clearly insufficient to act as a diagnostic assay as the two ELISAs with significant findings were only 20% sensitive for the detection of anti-HBRV Su antibodies in PBC patient samples. Further ELISA development is required to establish a relationship between PBC and anti-HBRV reactivity.

Preface

This thesis is an original work by Mark Kneteman. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Viral discovery in liver disease, inflammatory bowel disease and other idiopathic disorders", Pro00005105 February 14, 2012.

Some of the research conducted for this thesis forms part of an international research collaboration, led by Doctor Andrew Mason at the University of Alberta, with Michael Houghton and InBios International Inc. The human samples referred to in chapter 2.1 were collected by Center of Excellence for Gastrointestinal Inflammation and Immunity Research, while the preparation of the collected samples was done by myself. Dr. John law assisted in the design of the human betaretrovirus surface protein truncations and expression vectors referred to in chapter 2.2, while the cloning of the truncation and expression vectors was done by myself.

Dr. Guangzhi Zhang produced the human betaretrovirus surface protein expression construct referred to in chapter 2.13. Chelsea McDougall assisted in purification of the mammalian gp52 Su antigen referred to in chapter 2.14. All protein expression and purification referred to in chapter 2, excluding the assistance in purification of mammalian gp52 Su, was done by myself. All ELISAs and Western blots referred to in chapter 2, with the exception of the HBRV gp52 Su version 2 ELISA, were designed and done by myself. In regards to the ELISA version 2, I provided InBios with the ELISA version 1 protocol along with mammalian expressed HBRV Su, a serum panel of healthy control patients, PBC patients, and other liver disease patients. InBios modulated the protocol and contributed their own pool of healthy controls.

The data analysis in chapter 3 and concluding analysis in chapter 4 are my original work, as well as the literature review in chapter 1.

This thesis is an original work by Mark Kneteman. No part of this thesis has been previously published.

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In my pursuit of my Master's degree, I have been fortunate to have the guidance of my committee members and the support of friends and family.

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List of Abbreviations

Abbreviation	Meaning	Page Number
AMA	Anti-mitochondrial antibodies	2
APS	Ammonium persulfate	25
BCA	Bicinchoninic acid	30
BEC	Biliary epithelial cell	2
BSA	Bovine serum albumin	26
CV	Coefficient of Variance	43
DdH_20	Double distilled water	21
DMEM	Dulbecco's modified eagle medium	29
DMSO	Dimethyl sulfoxide	30
DNA	Deoxyribonucleic acid	8
DTT	Dithiothreitol	24
env	Envelope	8
E. coli	*Escherichia coli*	13
EIA	Enzyme immunoassay	12
ELISA	Enzyme-linked immunosorbent assay	12
FBS	Fetal bovine serum	29
FLAG	FLAG octapeptide	29
FPLC	Fast protein liquid chromatography	30
gag	Group-specific antigen	7
GST	Glutathione S-transferase	25
GWAS	Genome-wide association study	3
HBV	Hepatitis B virus	3
HBRV	Human betaretrovirus	1
HCV	Hepatitis C virus	3
HIS	Polyhistidine-tag	29
HIV	Human immunodeficiency virus	3
HLA	Human leukocyte antigen	3
IA	Immunoassay	12
IDD	Insulin-dependent diabetes	6

Abbreviation	Meaning	Page Number
IPTG	Isopropyl-beta-D-thiogalactopyranoside	24
LB	Lysogeny broth	23
MMTV	Mouse mammary tumor virus	5
NOD	Non-obese diabetic	5
NPV	Negative predictive value	67
NTPs	Nucleoside triphosphate	21
O.D.	Optical density	24
PBC	Primary biliary cirrhosis	1
PBS	Phosphate buffered saline	24
PCR	Polymerase chain reaction	10
PDC-E2	Pyruvate dehydrogenase complex E2	2
Pol	Reverse transcriptase and integrase	7
PPV	Positive predictive value	67
RIBA	Recombinant immunoblot assay	13
Sag	Superantigen	8
SDS	Sodium dodecyl sulfate	25
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	25
Su	Surface	1
TAE	Tris-acetate-EDTA buffer	20
TBS	Tris buffered saline	27
TEMED	Tetramethylethylenediamine	25
TMB	3,3',5,5'-Tetramethylbenzidine	29
UDCA	Ursodeoxycholic acid	2
XMRV	Xenotropic murine leukemia virus-related virus	10

Chapter 1: Introduction

1.1 Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease. It is thought to arise as a result of an environmental trigger in a genetically susceptible host. Our laboratory has characterized a human betaretrovirus (HBRV) in patients with PBC and also investigated the genetic factors that may predispose to PBC. Herein, we address the hypothesis that PBC patients develop humoral immunity to HBRV by studying serological responses to the Surface (Su) protein.

1.2 Primary Biliary Cirrhosis

PBC is characterized by chronic inflammation and granulomatous destruction of interlobular bile ducts (37). It is thought that the disease process is mediated by both cellular and humoral immune responses directed towards the cholangiocytes (13). Apoptosis exceeds proliferation leading to progressive intrahepatic bile duct loss. The loss of bile ducts results in cholestasis, liver fibrosis and cirrhosis (18). A proportion of patients develop liver failure and succumb unless they receive a liver transplant (37). Approximately 5% to 10% of liver transplants in North America and Europe are carried out for PBC (27).

PBC has a variable prevalence ranging from 1 in 2000 to 1 in 100,000, with cases in females being 10 times as prevalent as cases in males (37). The cause of the female preponderance of the

disease is unknown. Ursodeoxycholic acid (UDCA) is the only licensed therapy and it is effective in preventing deterioration in two thirds of patients. Some studies suggest that UDCA acts to partially restore alkalinisation and dilution of canalicular bile to help counteract damage by toxic bile acids (37). While disease progress can be slowed with prolonged treatment, reversal of ductopenia has not been achieved by UDCA therapy (27). Thus, novel therapies with potential to cure the illness are highly desirable.

PBC is considered an autoimmune disease because patients make anti-mitochondrial antibodies (AMA) against the dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex E2 (PDC-E2) (20). AMA also interact with the ketoglutaric acid dehydrogenase complex, the dihydrolipoamide dehydrogenase binding protein, and the branched-chain 2-oxo-acid dehydrogenase complex (33). Identification of AMA is possible years before PBC's clinical diagnosis and the autoantibody can be found in approximately 90% of PBC patients (13). In the nucleated cells of healthy individuals, PDC-E2 is found on the mitochondrial inner membrane (49). In PBC patients, PDC-E2 is found aberrantly expressed on the cellular membrane of biliary epithelial cells (BEC) (20). PDC-E2 is normally tolerated by the immune system, and exposure of aberrantly expressed PDC-E2 breaks tolerance to the mitochondrial protein leading to AMA production.

The autoimmune response in PBC includes T cell responses against mitochondrial antigens (33). PDC-E2 reactive B cells, CD8+ T cells, and natural killer cells are more plentiful in the liver than peripheral blood. Even AMA negative PBC patients have been found to have CD4+ T cells that are autoreactive. As such, it has been suggested that the initial immunological insult may be directed to PDC-E2 by a multi-lineage immune response constituted by autoreactive autoantibodies, CD4+ and CD8+ T cells, and B cells (33).

Current evidence suggests PBC is a complex disease resulting from a combination of genetic and environmental factors. PBC is likely caused by multiple genetic risk factors that predispose to infection or a xenobiotic pathology from an unknown environmental agent. Plausible environmental risk factors include exposure to xenobiotics and infectious agents (37).

1.3 Genetic predisposition to PBC

The role of genetics in PBC has been supported by familial PBC rates, twin studies, and genome wide association studies. There is a ten-fold increased risk in family members of PBC patients of developing the disease (18). However, as concordance rates for twin studies fall short of 100% (18), environmental factors must also play a role in PBC etiology. Genetic factors that likely influence PBC include some alleles of the human leukocyte antigen (HLA) loci DR and DQ, as well as other loci implicated in immune function such as genes encoding IL12A, TNF-α, CTLA4, and IL12RB2 (37). Since most genetic risk associated with PBC is associated with immune function, PBC is likely preceded by a genetic predisposition for a dysfunctional immune responses to specific infectious agents or xenobiotics (37). Environmental insults acting on a dysfunctional immune system then lead to inadequate immune clearance and autoimmunity.

Genome wide association studies (GWAS) have demonstrated that Human leukocyte antigen (HLA) loci have the strongest associations to PBC of genetic regions (18). The HLA region contains loci associated with inflammatory responses. HLA also influences self-tolerance and immune responses as they encode cell surface molecules that mediate key immunological events through their role in peptide presentation. HLA alleles linked to PBC susceptibility may produce proteins ineffective in antigen presentation, leading to failure in self-peptide presentation to developing T cells and negative selection failure. Thus, some HLA alleles may enhance immunogenicity by influencing T cell repertoire and thereby enhancing autoimmune activation. HLA allele variants protective against PBC are also associated with protection against infectious agents such as HCV, HBV, HPV and HIV (18). The *HLA DRB1*08* allele is associated with PBC susceptibility while *DRB1*11* and *DRB1*13* are associated with strong protection against PBC. While the associations discussed are established, there is little understanding of the causative link between particular genetic polymorphisms and PBC.

1.4 Environmental Factors and PBC

Xenobiotics, and infectious agents have all been supported as plausible environmental insults acting as triggers for PBC. Efforts to establish a single causative agent of PBC have not yet succeeded. Pollutants have been linked to PBC via studies establishing a relationship between clustering of PBC prevalence and toxic waste site locations (37). However, disease clusters

identified through epidemiological studies could also support infectious factors, as studies identifying spatial and temporal clustering of PBC are compatible with vector or person-to-person transmission (19). A relationship has also been found between mean daily airborne pollutant concentrations and individuals listed for liver transplantation (37). Cigarette smoking is consistently associated with a worse prognosis in patients with PBC.

It has been suggested that xenobiotics may alter or complex with proteins, either self or foreign, to trigger immunity via cross-recognition of the self-antigen (45). Xenobiotic based hypotheses for PBC focus on molecular mimicry due to modifications of lipoylated PDC-E2 leading to autoimmunity. The lipoic acid residue of the PDC-E2 epitope is crucial to immune recognition; as it is exposed on the complex's exterior, the residue presents a target for modification by a candidate xenobiotic. Studies with recombinant lipoylation enzymes have suggested that xenobiotics can be included in the PDC-E2 complex in place of lipoic acid. If occurring in an individual with a genetic background permissible for PBC, xenobiotic modification of PDC-E2 via the inclusion of a xenobiotic could result in tolerance breakdown and production of autoantibodies cross-reactive with normal PDC-E2. Tentative xenobiotics are 2-ocyynoic acid and 2-nonynoic acid, which are found in various cosmetic products.

However, most evidence pertaining to xenobiotics in PBC etiology focuses on establishing whether the mechanism is conceptually plausible. Little evidence exists of a xenobiotic being associated with PBC-like disease *in vivo*, with the exception of xenobiotic animal models of PBC that exhibit significant departures from clinical PBC. Future studies should collect evidence of a consistent association between PBC development and xenobiotic exposure as previous results considering xenobiotics in make-up have been inconsistent.

Bacteria such as *Novosphingobium aromaticivorans* and a human betaretrovirus (HBRV) are infectious agents suspected in PBC etiology (45). Support for an infectious etiology is seen in liver transplant recipients because recurrent PBC is earlier and more severe with stronger immunosuppressive therapy following transplantation (31). One suggested mechanism of autoimmune pathology is molecular mimicry leading to loss of tolerance. Bacteria exhibit cross reactivity between PDC-E2 epitopes, which is a highly conserved protein. It is notable that PBC patients exhibit higher rates of urinary tract infections. The bacterium *Novosphingobium aromaticivorans* has been suggested as a possible candidate trigger for PBC (45). It is

ubiquitous, but non-pathogenic, and thus could plausibly trigger rare pathology in individuals with permissive genetic backgrounds. While AMA react with the bacterium's two 47 to 50kD lipoylated proteins, the bacteria is not detected in PBC liver and not specifically associated with PBC, regardless of the serological reactivity.

1.5 Viral Etiology for PBC

A viral infection has been proposed as a candidate agent in patients with PBC (49). While this hypothesis remains controversial, it is notable that betaretrovirus infection of biliary epithelial cells (BEC) results in surface up-regulation of PDC-E2 or a protein capable of cross-reactivity to AMA (49). The betaretrovirus is suggested to contribute to breaking tolerance by exposure of normally sequestered PDC-E2.

The betaretroviral hypothesis is supported by evidence from PBC patient clinical samples showing presence of viral particles in biliary epithelial cell (BEC) cultures, viral nucleic acid in liver draining lymph nodes, and serological responses to retroviruses (28, 49). A spontaneous mouse model of PBC, the NOD.c3c4, which makes AMA and develops cholangitis has evidence of infection with the highly related betaretrovirus, mouse mammary tumor virus (MMTV). The role of betaretroviral infection in PBC remains controversial because others have been unable to reproduce the original findings, although no group has investigated the presence of virus in PBC patients' draining lymph nodes.

Definitive establishment of an environmental trigger for PBC would be strongly supported by the discovery of a general underlying genetic background for PBC susceptibility, and subsequent development of a representative mouse model. PBC animal models of both bacterial and retroviral infection exist, but both exhibit significant departures in pathology from human PBC. While mechanistically sound, these infectious agent hypotheses require an appropriate model to exhibit their role in instigating pathology *in vivo*.

1.6 Animal Models and PBC

In induced PBC models, suspect environmental agents are introduced to subject animals, and subsequent pathology is monitored. In spontaneous mouse models, functional alleles are "knocked-out" or replaced by breeding in immune deficient strains. These models offer the

closest approximation to human disease in an experimental setting. All current mouse models of PBC exhibit significant differences in pathological characteristics from human PBC. A best-fit model of PBC would be expected to exhibit the characteristics: CD4+, CD8+, and B cell liver infiltrates, AMA against PDC-E2, liver granuloma and fibrosis, PBC-like serological markers of cholestasis and elevated pro-inflammatory cytokines, female preponderance, PBC-like liver histology, and characteristic T regulatory cell alterations.

The NOD.c3c4 strain was produced from the non-obese diabetic (NOD) mouse model. The NOD.c3 mouse is a NOD mouse with an insulin-dependent diabetes (Idd) resistance allele from the C57BL/6 mouse strain on only chromosome 3 (23). These mice exhibit no autoantibody production, but have lymphocytic liver infiltration. NOD.c4 mice have the same resistance allele, but only on chromosome 4, and exhibit autoantibody production without lymphocytic liver infiltration. The NOD.c3c4 mouse has both Idd resistance alleles and does not develop diabetes but spontaneously exhibits polycystic biliary tract disease reminiscent of PBC (23). Anti-PDC-E2 AMA, lymphocytic peri-biliary infiltrates, hepatomegaly, and progressive biliary obstruction characterize the NOD.c3c4 disease phenotype (8). While the model lacks T regulatory cell alterations and hepatic B cell infiltrates (23), these are relatively limited deviations from PBC pathological characteristics. The observation that manipulation of limited genes can lead from development of one autoimmune phenomena to another supports that similar dysfunction of biochemical pathways could result in divergent disease phenotypes. Furthermore, it is the interaction between the NOD genome and the Idd resistance genes resulting in a novel disease phenotype, not the removal of functional genes.

Our lab has shown that the NOD.c3c4 mouse model has infection with MMTV (50). The NOD.c3c4 has evidence of MMTV capsid and surface protein, and a PDC-E2 cross-reactive protein, on its biliary epithelium (50). It is thought that MMTV is endemic in this strain as an endogenous retrovirus that leads to exogenous infection with passage of MMTV in breast milk in the neonatal period. The hypothesis for AMA production is that MMTV exposure leads to PDC-E2 tissue expression leading to breakdown of immune tolerance to self, expressed in the context of infection. Thus, the model may potentially offer an example in which tolerance breakdown results from a combination of retroviral exposure and immunogenetic susceptibility. Of note,

antiviral treatment inhibiting MMTV infection in the NOD.c3c4 directly impacts on the cholangitis, suggesting that the viral infection is integral to the disease phenotype.

Similar observations have been made in other spontaneous mouse strains of autoimmune biliary disease that make AMA (50). The *IL-2Rα*$^{-/-}$ mouse model develops serum AMA against PDC-E2 between the ages of 4 to 22 weeks and diffuse inflammatory disease in the liver and GI tract. Further experiments including CD4 and CD8 knockouts suggest that, in the context of T regulatory cell deficiency, biliary ductular damage is mediated largely by CD8+ T cells. The T cell TGF-β receptor II dominant-negative mice also develops AMA, liver and gastrointestinal inflammatory disease. Both of these models express high levels of MMTV associated with aberrant expression of PDC-E2 consistent with the idea that viral infection helps break tolerance to self-proteins (50).

Animal models present a tool with which hypotheses on environmental and genetic factors contributing to PBC can be tested. While inbred genetically modified mice are far from representative of the human population, they are the best *in vivo* approximation of disease conditions available for empirical testing. These models can be considered "immune deficient" as they display numerous immune related defects. Accordingly MMTV infection may become activated in the absence of adequate immune surveillance. However, these models fail to reflect the whole hypothesis of PBC development. The NOD.c3c4 mouse is a possible candidate for a comprehensive model in that it possibly exhibits infection by an environmental agent in a genetically predisposed host and the pathological characteristics of the model largely reflect those in PBC. However, they do not necessarily reflect the genetic risk factors associated with PBC, namely abnormal IL-12 axis (37). With further understanding of PBC's underlying genetics, models can be built for further testing with environmental agents to find what combination of genetic and environmental conditions will result in PBC-like disease reflecting human PBC development and progression.

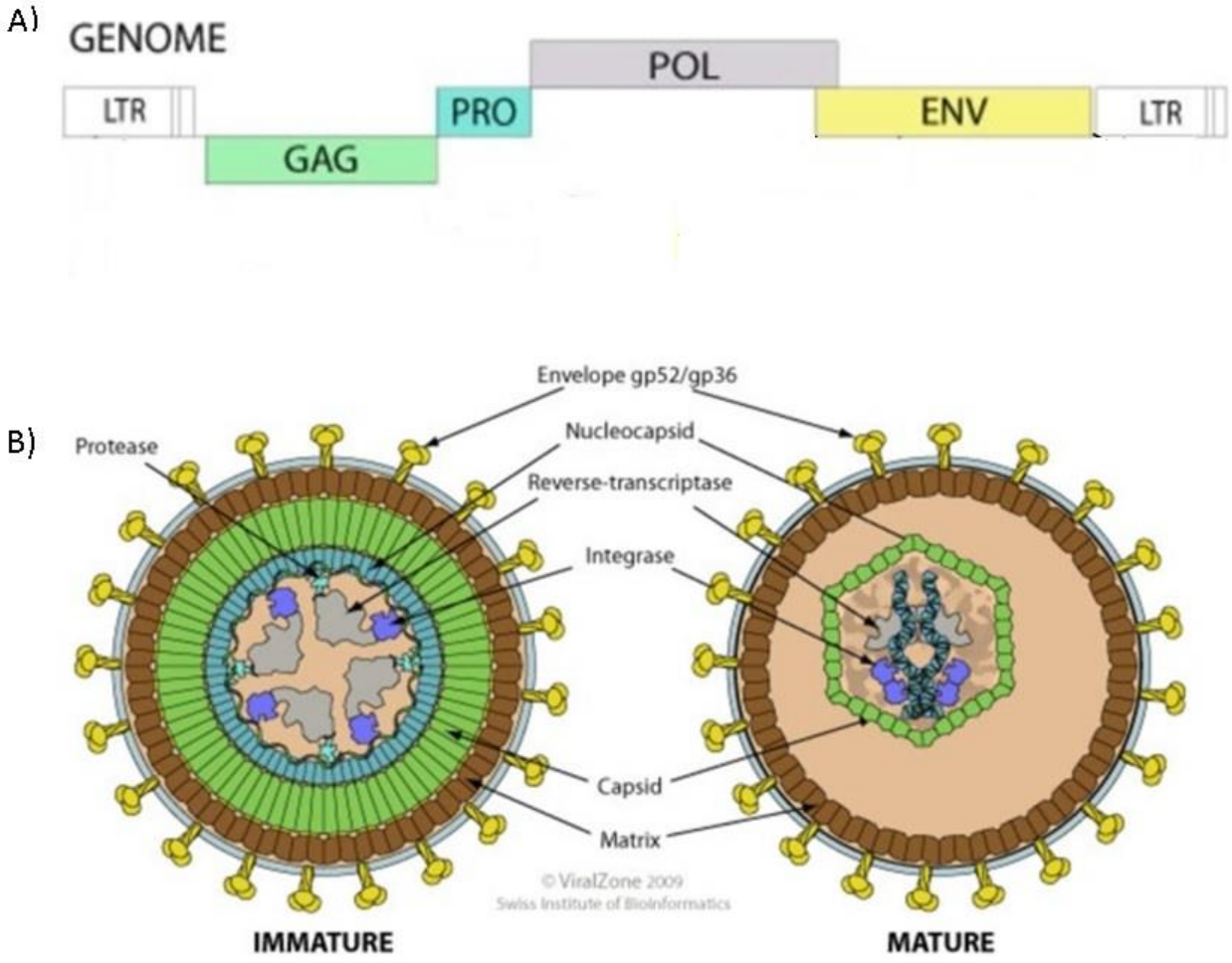
1.7 Mouse Mammary Tumor Virus

Mouse mammary tumor virus (MMTV) is both an exogenous and endogenous betaretrovirus in mice. The viral genome generates 5 transcripts via alternative splicing. *Gag*, *dut-pro*, and *pol* genes are produced from the full length unspliced mRNA, but the *dut-pro* and *pol* genes are

translated in different reading frames from the *gag* (40). The full length mRNA can also be packaged into virions as the viral genome. The Capsid (CA) and Nucleocapsid (NC) proteins, as well as proteins of unknown function, are produced by processing of the Gag translational product by the viral protease encoded by Pro. Dut-Pro and Pol are translated as a polyprotein. Pro codes for the viral protease and Dut for a dUTPase with an unknown biological role. Pol codes for the reverse transcriptase (RT), that produces DNA from the virion's RNA genome, and integrase, which allows integration of the RT produced DNA into the host genome (40).

The envelope gene is translated from a singly spliced mRNA that is cleaved by host enzymes, producing the Surface (Su) and Transmembrane (Tm) domains of the Envelope protein (Env) (40). Virion to cell surface receptors mediate cell entry and are bound by Env. Su provides the receptor binding site and Tm mediates virion- cell membrane fusion. The Su protein binds the MMTV entry receptor, transferrin receptor 1. Env has been shown to use toll-like receptor 4 to activate antigen presenting cells, such as B cells (40). The MMTV *sag* gene produces a superantigen, which is essential to host infection and disease (7). Conventional antigen stimulates only 0.01-0.001% of T cells while the Sag protein can stimulate up to 10%.

Figure 1: Schematic of MMTV Genome Organization and Virion Particle.
A) The MMTV genome contains *gag*, *pro*, *pol*, *env*, and *sag* (not pictured) genes (7). The *gag* encodes the Capsid, Nucleocapsid, Matrix and other proteins of unknown function; *pro* encodes the viral protease; *pol* encodes the reverse transcriptase and integrase enzymes and *env* encodes for the signal peptide, Surface domain (gp52 Su), and Transmembrane domain (gp36) of the Envelope protein; *sag* encodes the viral superantigen B) The viral particle diagram depicts the Envelope protein exposed from the particle with the Matrix beneath (24). Within the Capsid, the viral RNA in association with the viral integrase and reverse transcriptase enzymes.



MMTV can be acquired vertically by inheriting endogenous copies of provirus, or horizontally by consumption of virus present in milk of infected mothers (40). In healthy strains of mice, endogenous proviruses do not produce functional virus. Virus is transmitted to pups via infected milk from the mother (7). The first cells infected in a mouse are the dendritic cells in the Peyer's patches and small intestine (40). Env interaction with Toll-like receptor 4 triggers migration of the infected dendritic cells to nearby lymph nodes. B cells are also infected, and subsequent Sag-

mediated T cell stimulation leads to a proliferative B cell response, which expands the pool of susceptible and infected B cells (7). The mammary epithelium is infected by the transmission of infected B cells that travel to the mammary gland.

Transformation of MMTV infected cells depends on proviral integration near cellular proto-oncogenes (40). As virus integration seems to be non-site specific, higher viral burden relates to higher risk of transformation.

MMTV induces T-cell lymphomas and mammary tumors in mice (7). MMTV, or an MMTV-like virus, has also been extensively investigated as a possible human infectious agent involved in breast cancer and more recently in PBC (16). It is suspected that exposure to mouse deposits could be a means by which MMTV infection of humans occurs leading to breast cancer (25). This is supported by correlational epidemiologic evidence that regions highest in breast cancer prevalence correlate with geographic distribution of *Mus musculus*, a mouse species thought to shed more MMTV virus than other species.

Groups have succeeded in identifying MMTV sequences in human mammary carcinomas. Proviral sequences were exclusively identified in breast cancer patient samples, but not non-malignant samples (16). Furthermore, evidence has been found of nucleic acid sequences resembling MMTV in pre-invasive lesions of human breast tissue, such as ductal carcinoma in situ, via chromogenic in situ hybridization and florescent nested PCR (25). No MMTV-like sequences were found in control samples. Despite these findings, difficulties replicating PCR based evidence for MMTV infection in human patients has led to ongoing scepticism of MMTV as a human pathogen. The requirement of PCR or nested PCR to establish presence of viral DNA suggests that MMTV infections in humans are likely low-level (43). As such, the biological role in human infection may differ from that in mice. It is also possible that findings have been due to contamination, such as that which occurred in studies for xenotropic murine leukemia virus-related virus (XMRV) in association with prostate cancer.

It was long posited that MMTV was unable to actively infect human tissue, leading to further scepticism of its role as a human pathogen. However, Indik et al. succeeded in exhibiting that both wild-type and genetically modified EGFP labelled MMTV were capable of infecting a number of cultured human cells (16). The infected cells were subsequently capable of producing infectious virions.

1.8 Human Betaretrovirus

While breast cancer has been the focus of possible infectious consequences of MMTV infections in humans, PBC provides another possible association with the virus. PBC patient sera is reactive to HIV-1 proteins and human intracisternal A-type particles, suggesting involvement of a virus in the disease process (27). Furthermore, electron microscope studies have found virus-like particles in PBC patient BEC (49).

Evidence has been established suggesting association between PBC and an MMTV-like betaretrovirus, the human betaretrovirus (HBRV). Proviral DNA with >90% nucleotide similarity to MMTV was cloned via nested PCR from two PBC patients' perihepatic lymph nodes (49). MMTV and human breast cancer betaretrovirus amino acid sequences exhibited 93 to 97% and 93 to 99% similarity to the betaretrovirus Env and p27 Ca proteins. This putative virus was dubbed HBRV, although it is unclear whether the virus represents a zoonotic MMTV infection, or a distinct virus (49).

Subsequent immunochemistry and RT-PCR studies found the perihepatic lymph nodes of 75% of PBC patients to be positive for viral protein and RNA compared to <20% of controls (49). Preliminary studies using linker-mediated PBC have demonstrated that up to 70% of PBC patients harbor HBRV in biliary epithelial cells (17). Demonstration of viral integration sites in the host genome is considered the strongest evidence of retroviral infection (10). In Western blot studies, a majority of PBC patients exhibited antibody reactivity to MMTV Envelope proteins as well as AMA reactivity (28). Furthermore, co-culture of normal BEC cells with PBC patient homogenized lymph nodes resulted in the BEC exhibiting an aberrant expression of PDC-E2-like protein, one of PBC's phenotypic manifestations (41). The transmissible factor that promoted the PBC phenotype was found to be gamma radiation sensitive, a finding consistent with an infectious process (49).

However, many of the critiques that apply to MMTV's involvement in breast cancer also apply to the hypothesis that HBRV is involved in PBC. Salmons and Gunzburg suggest that developing a sensitive and specific immunological assay for anti-MMTV antibodies is an important step in establishing an association between breast cancer and MMTV (43). This logic could also apply to HBRV association with PBC. They recommend studies using standardized recombinant MMTV antigen should be undertaken in a similar manner to standard HIV diagnosis, where

enzyme-linked immunosorbent assay (ELISA) technique is followed with confirmatory Western blot of positive samples to confirm recognized protein size reflects that of authentic viral protein (43).

1.9 Viral Diagnostics

For infectious retroviral agents, standard diagnostic approaches utilize a screening immunoassay (IA), such as ELISA, followed by a second confirmatory method to use on repeatedly reactive samples. For example, third generation HIV testing depended on an IA-Western blot algorithm (4). Samples screened via IA were then confirmed using western blot, which allowed visualization that the reactivity noted in the IA was specific to the HIV antigen. Current generation testing involves an initial IA followed by an HIV-1/HIV-2 antibody differentiation test, and possibly an HIV-1 nucleic acid amplification test in the case of inconclusive or negative results (4). Some of the various antigenic markers used in enzyme immunoassays include HIV-1 gp41, gp160, and p24 and HIV-2 gp36 (34). P24 is the Ca protein, gp160 is the Env region product, and gp41 is the Transmembrane protein (7).

There are various methods for human T-cell leukemia virus (HTLV) types 1 and 2, but initial testing can consist of enzyme-linked immunoassay (EIA) followed by confirmation using Western blotting, which allows discrimination between the two viral strains (12). HTLV-1 can also be tested using particle agglutination in place of EIA. Potential confirmatory methods also include radioimmunoprecipitation assay and immunofluorescence assay. Gp46 and p24 are two HTLV antigens used in diagnostic ELISAs and Western blots (47). Gp46 is the Su protein, p24 is the Ca protein (7).

Anti-HCV antibodies are used to determine hepatitis C virus exposure, with HCV RNA used via RT-PCR to determine infection (1). First generation enzyme immunoassay displayed poor sensitivity and specificity using a recombinant HCV peptide (1), which was superoxide dismutase-HCV polypeptide, the non-structural C100-3 peptide (32). Using a 1st generation assay for anti-HCV, within 6 weeks of illness onset 45% of patients with HCV infection produced anti-HCV antibodies, compared to 68% of patients followed for upwards of 6 months (2). Using this peptide results in false-positives when probing for antibodies in the sera of

patients with a variety of non-HCV liver disease conditions (1). The assay preferentially detects antibodies from chronically HCV infected patients.

The second generation HCV enzyme immunoassay utilized the C100-3 peptide as well as two non-structural region antigens and HCV core antigen (1). Probing sera with multiple antigens achieved superior specificity and sensitivity. Utilizing the second generation HCV enzyme immunoassay, Alter et al. found 93/106 (87.7%) patients with HCV infection exhibited anti-HCV antibodies by serum (3). Of 13 patients that tested negative for anti-HCV, 9 displayed evidence of HCV infection via HCV RNA amplified via PCR and 9 were positive for anti-HCV core antigen by fluorescent-antibody blocking assay. 91 of 93 patients initially positive for anti-HCV exhibited persistent anti-HCV. (3).

The third generation further added another non-structural HCV antigen, further improving the ability of the assay to detect HCV infection (1). The third generation HCV EIA was found to possess a sensitivity of 98.1% and specificity of 98.4% compared to the second generation HCV EIA, which exhibited a sensitivity of 85.6% and a specificity of 98.4%. Using recombinant immunoblot assays (RIBA) to confirm discordant results between second and third generation HCV EIA tests, the third generation HCV EIA agreed with RIBA testing for 87.5% of discordant patients while the second generation HCV EIA agreed only 12.5% (1).

Antiviral antibodies are more variable in the case of HCV infection compared to HBV infection (39), but remain useful for diagnostic testing. Also, unlike anti-HBV antibodies, HCV antibodies may decline beyond the limits of detection 10-20 years post resolution of infection. While HCV Envelope proteins E1 and E2 are not utilized in diagnostic assays, they can elicit antibody responses from patients (15). Insect cell produced E1 exhibits 10-40% reactivity with sera while *E. coli* produced E1 reacted with up to 93%. *E. coli* and baculovirus system expressed E2 exhibits reaction with ~70% of chronic HCV patient sera. The omission of HCV E1 and E2 from diagnostic antigen panels may be one of convenience, as expression and purification of the glycosylated antigens is challenging.

Presence of HBV infection is most accurately tested by sensitive PCR testing for viral DNA (48). Serological detection of HBV infection is used for clinical diagnosis via probing for HBV antigens and antibodies against HBV antigens. The specific antibodies of interest are anti-HBV surface antigen (anti-HBs), anti-HBV core antigen (anti-HBc), and anti HBV e antigen (anti-

HBe). Initial infection is indicated by anti-HBc IgM (39), while, anti-HBs and anti-HBe antibodies indicate reduction of viral replication and assessment of immunity (48). Historically, HBe antigen was used as a surrogate marker to indicate an ongoing replicative infection. Chronic HBV infection is associated with persistent anti-HBc, where anti-HBs either indicates a state of viral clearance or vaccination with HBsAg. Anti-HBV antibodies can decrease to undetectable levels in some situations, but immunological protection is not necessarily lost (6). Anti-HBc antibodies consist of IgM and IgG. During acute HBV infection, anti-HBc IgM is detectable for 6 months before declining. Anti-HBc can persist indefinitely.

Given the models for investigating other viruses involved in human disease, it would be beneficial to examine whether similar diagnostic paradigms could be developed to examine human serum samples from individuals with signs of HBRV infection.

1.10 Hypothesis

We will address the hypothesis that patients with PBC make humoral immune responses to HBRV Env proteins. Accordingly, we aim to develop a sensitive assay for detection of HBRV Env serological reactivity to evaluate immune responses to HBRV or lack thereof, in patients with PBC. In order to test our hypothesis, we will design a sensitive and specific serological assay to detect antibody reactivity to HBRV Su protein.

1.11 Implications and Importance of Investigation

Large scale immunoassay investigation of humoral immunity in PBC patient and control serum will provide evidence as to whether PBC patients produce humoral immune responses to HBRV proteins. By extension, they would also support whether or not an MMTV-like virus can infect humans and would suggest whether or not HBRV has a role in PBC's etiology.

Whether or not PBC patients produce anti-HBRV antibodies has not been firmly established in the literature, as previous studies have focused specifically on Western blot technique utilizing MMTV lysates (28, 46). As such, Western blot and ELISA studies utilizing recombinant HBRV Su would contribute to the understanding of the relationship between HBRV and PBC. Furthermore, the establishment, or lack thereof, of an association between anti-HBRV antibody production and PBC diagnosis may influence the direction of further investigations of the

etiology of PBC by either supporting or not supporting the hypothesis of the retroviral etiology of PBC.

Previous studies on HIV (22) and HTLV-1 (44) have exhibited that individuals with retroviral infections produce humoral responses to viral proteins. Successful detection of antibodies specific to viral antigens in PBC patient sera will be indicative of infection. Lack of anti-HBRVantibodies would suggest either that the assay was not sufficient to successfully detect antibodies that were present, or would suggest no viral infection of patients, unless evidence of a viral immune-evasion mechanism could be established.

Recombinant HBRV Su proteins will be used to establish an indirect enzyme linked immunosorbent assay (ELISA) to screen PBC patient sera for prevalence of HBRV Su specific antibody. As HBRV and MMTV exhibit few functional and genetic differences (49), positive results in immunosorbent assays would also support claims that MMTV is capable of infecting humans. The use of Western blot to confirm positive samples would lend greater credibility to the assay's findings.

An established ELISA against anti-HBRV antibodies could potentially be used to screen the general population to test prevalence of HBRV infection in healthy individuals. Large epidemiological studies could also elucidate the role of HBRV in chronic liver disease development and to assist in determining HBRV's contribution in PBC's etiology.

If there is a strong association between anti-HBRV antibodies and diagnosis of PBC, and further studies reveal a subset of healthy individuals with anti-HBRV antibody production, humoral immunity to HBRV may provide a potential predictor of individuals at risk for developing PBC. Identifying individuals infected with HBRV could allow the formation of cohorts to be followed prospectively in order to examine the possible relationship between infection and disease. Breast cancer could also present a disease of interest as MMTV was long studied in association with breast cancer.

The possibility that PBC could be caused by a zoonotic infection suggests that a large number of healthy individuals may be infected with HBRV. A subset of these individuals with a predisposing genetic background may go on to develop PBC. Developing an assay capable of screening large numbers of serum samples for evidence of HBRV infection, in the form of

humoral responses to viral antigens, would allow for identification of an at risk population for PBC. If HBRV infection is conclusively shown to be involved in PBC's etiology and progression, an assay capable of identifying infection could allow for infected individuals to be monitored long term, possibly allowing earlier treatment commencement at the first signs of disease.

Finally, if the results of this study were to contribute to solidifying a role for retroviral infection in PBC, then research into the use of retroviral specific treatments for PBC may lead to more treatment options in the future. Small scale studies examining the utility of antiretroviral therapy in PBC treatment have already been undertaken, but larger placebo-controlled trials would be necessary to fully assess this treatment option (26).

Chapter 2: Materials and Methods

2.1 Human Samples

A serum panel was gathered to examine reactivity of PBC, control liver disease, and healthy control serum samples against *E. coli* and mammalian produced gp52 Su in Western blots and ELISAs (Table 1). Samples were largely collected from The Canadian BioSample Repository (***Edmonton, AB, Canada***), including PBC patient samples and other liver disease patient samples. The Mason lab healthy controls were contributed by the BioSample Repository and during our lab's blood drive, as well as through kind gifts from Dr. Luiz Lisboa and Dr. Amir Landi. Healthy control serum from breast cancer studies was also provided by Dr. John Mackey.

Table 1: Serum Panel for Immunoassay Studies

Diagnosis	Total
PBC	124
Multiple Diagnoses with Primary Diagnosis as PBC	10
PBC Transplant	8
Granulomatous Hepatitis	1
HCV and Multiple Diagnoses with Primary Diagnosis as HCV	9
HBV and Multiple Diagnoses with Primary Diagnosis as HBV	2
NASH and Multiple Diagnoses with Primary Diagnosis as NASH	18
PSC and Multiple Diagnoses with Primary Diagnosis as PSC	28
NHS Healthy Controls	24
Mason Lab Healthy controls	48
Breast Cancer Healthy controls	102

2.2 Human Betaretrovirus Su Truncations from *E. coli*

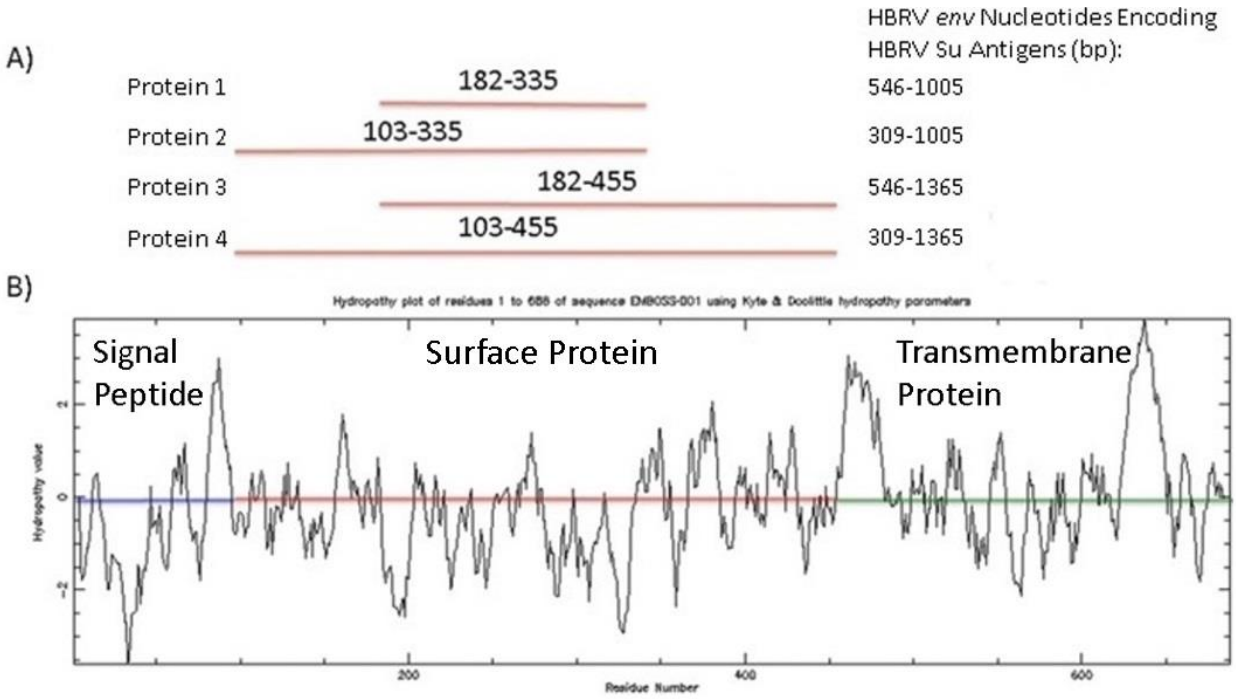
Published sequence of Human Betaretrovirus reported by Xu et al. were utilized by John Law to create a consensus *HBRV env* sequence from the BLASTN database (49). *HBRV env* containing plasmid was provided as a gift by John Law. The consensus sequence was used with MacVector (***Version 10.6.0, MacVector Inc.***) to design 4 DNA constructs for production of truncated HBRV proteins. Studies began with production of the full length *HBRV su* and the three HBRV su truncations from the cloned HBRV genome via commercial primers. Internal primers were first used to correct any differences between the HBRV genome template and a reported HBRV genome sequence.

Truncation lengths and positions were chosen according to which Su regions would most likely produce hydrophilic proteins when expressed. The full-length HBRV gp52 Su was encoded by base pairs 309-1365 of the *HBRV env* gene (Construct 4), corresponding to amino acids 103-455 referred to as Protein 4 (Figure 2). The three HBRV su truncations encoded the smallest and most hydrophilic region of the Su (Construct 1), the Su from the signal peptide terminus to the end of Construct 1 (Construct 2), and the Su from the beginning of Construct 1 to the beginning of the Transmembrane protein (Construct 3).

Construct 1 encoded base pairs 546-1005 of the *env* gene, corresponding to amino acids 182-335 (Protein 1). Construct 2 contained base pairs 309-1005 and corresponded to amino acids 103-335

Figure 2

Hydropathy Plots Related to HBRV Su Proteins Expressed from HBRV su Constructs. A) Four proteins were designed from the HBRV *env* sequence. Construct 1 encoded base pairs 546-1005 of the *env* gene, corresponding to amino acids 182-335 (Protein 1). Construct 2 contained base pairs 309-1005 and corresponded to amino acids 103-335 (Protein 2). Construct 3 encoded base pairs 546-1365 and corresponded to amino acids 182-455 (Protein 3). Proteins were selected based on minimizing hydrophobicity of each protein to maximize likelihood of expressing soluble antigen. Protein 1 consisted of the most hydrophilic region of the HBRV Su. Protein 2 was the second most hydrophilic region, and from the beginning of the HBRV Su to the beginning of a hydrophobic region at amino acid 335 of the HBRV Env. Protein 3 extended from the beginning of the hydrophilic Protein 1 to the end of the HBRV Su. Protein 4 consisted of the entire HBRV Su. B) Hydropathy plot of HBRV Env amino acid residues. More positive values signal greater hydrophobicity. Selection of HBRV Su proteins emphasized areas of the HBRV Env possessing more negative values, and thus higher hydrophalicity. The hydropathy plot of HBRV Env was produced using Kyte and Doolitte hydropathy plot **[MacVector 10.6]**.



(Protein 2). Construct 3 encoded base pairs 546-1365 and corresponded to amino acids 182-455 (Protein 3).

An aliquot of HBRV DNA sequence existed in the lab with a single nucleotide polymorphism differing from the published sequence. We designed 6 primers for use in PCR experimentation

from Integrated DNA Technologies Inc. (***San Diego, CA, USA***). Primers 1, 2, 5 and 6 were designed to amplify the whole *HBRV su*, then to later create DNA truncations for the 4 protein constructs (Table 1). Primers 3 and 4 were used to correct the lab's *HBRV su* sequence to the published sequence.

2.3 Production of Full-Length *HBRV su*

The full-length *HBRV su* was combined with primers to produce two DNA truncations that could then be used in PCR experiments to produce amplified full-length *HBRV su,* and to repair a discrepancy between the Mason lab *HBRV su* and a published *HBRV su* sequence. Primers 1 and 4 were used in a mix to generate a 200bp DNA truncation at the 5' end of the *HBRV su* sequence while primers 3 and 6 were used to generate an 800bp DNA truncation at the 3' end (Table 2). Primers 3 and 4 overlapped with respect to the full HBRV sequence because a single nucleotide polymorphism was present in our lab's *HBRV su* compared to the published HBRV sequence. At the 186 bp position relative to the beginning of the construct 4, the full length *HBRV su*, a cytosine had to be changed to an adenine. As such, by producing two distinct DNA truncations using primers overlapping with the single nucleotide polymorphism, it was possible to produce two partial HBRV su constructs corrected to the published sequence, which could then be used in PCR to produce the correct full-length construct.

Recipe for 1.5% agrose gel used consistently in PCR experiments was 50mL TAE and 0.75g agrose (***EM Science, Gibbstown, NJ, USA***), boiled in a microwave under high power for 2x 30 seconds. 1:10 000 SYBR© safe DNA gel stain (***Invitrogen™, Carlsbad, CA, USA***) was added once the gel solution had cooled. The gel was poured into a 50 mL mould with an 8 well comb in place. The gel was run on a Power Pac 3000 (***Bio Rad Laboratories Canada Ltd., Mississauga, ON, Canada***) at 150V for 30' or until bands had separated distinctly.

Table 2: Primer Sequences for Constructing HBRV su Truncation Expressing Plasmids

No.	Primer sequence (5' to 3') *	Restriction enzyme
Primer 1 FW	CAG GAA TTC CC T GGG CTT ACC TAC CT	EcoRI
Primer 2 FW	CAG GAA TTC CCT CTG ATA CAC CCA CG	EcoRI
Primer 3 FW	TCC TTA TCC TTT TCT ACC CCC	N/A
Primer 4 RV	GGT AGA AAA GGA TAA GGA AAA GCA AA	N/A
Primer 5 RV	GCT CGA GTC AAT CTC TAT CAT TGG GA	XhoI
Primer 6 RV	GCT CGA GTC AGG CTC GAA TTA AAT CT	XhoI

* Restriction sites underlined.

PCR tube mix was prepared on ice. A 10mM dNTP mix was created from diluting equal parts 100mM aNTP, cNTP, tNTP, and gNTP (***Life technologies Inc., Burlington, ON, Canada)*** in ddH_20. Two 50µL PCR mixes were composed of: 100ng of *HBRV su* template, 5µL 10x PCR buffer (***Life technologies Inc.***), 1µL 10mM dNTPs, and 2µL 50mM $MgSO_4$ (***Life technologies Inc.***). Then 3µL of primers 1 and 4 were added to one mix while 3µL of primers 3 and 6 were added to the other. Both primers were at a concentration of 10pmol/µL. 0.2µL Taq DNA Polymerase (***Life technologies Inc.***) was added to both. DdH_20 was added to bring the final volume to 50µL. The PCR protocol was completed on a mastercycler© gradient (***Eppendorf Canada, Mississauga, ON, Canada***). The PCR program began with a 2 minute cycle at 94°C. A trio of cycles (94°C for 30s, 50°C for 30s, 68° for 30s) was repeated 30 times. The program ended with a 5 minute cycle at 68°C.

The PCR products were mixed with 6x DNA Loading Dye (***Thermo Fischer Scientific, Burlington, ON, Canada***), then loaded on a 1.5% agrose gel and analyzed. Successful production of the DNA truncations was confirmed via visualisation using a gel doc (***Bio Rad Laboratories Canada Ltd., Mississauga, ON, Canada***) and subsequent analysis using Quantity One software (***Version 4.6.7, Bio Rad Laboratories Inc.***). The 200bp and 800bp truncations were then gel purified using a QIAquick© Spin Kit (***Qiagen, Missassauga, ON, Canada***) according to the manufacturer's instructions in their microcentrifuge protocol.

2.4 Production of Full-Length *HBRV su* Template

The overlapping 200bp and 800bp HBRV su DNA truncations generated in the previous experiment were used as templates to generate full-length *HBRV su* template. One master mix was prepared on ice including: 2µL of the gel purified 200bp and 800bp template each (a 1:1 ratio was determined to afford the most promising results compared to 2:3 and 1:3, 200bp:800bp), 25µL 10x buffer (***Life technologies Inc.***), 5µL 10mM dNTPs, 10µL 50mM $MgSO_4$ (***Life technologies Inc.***), 15µL each of primers 1 and 6. Both primers were at a concentration of 10pmol/µL. 0.2µL Taq Polymerase (***Life technologies Inc.***) was added to both. DdH_20 was added to bring the final volume to 50µL. The PCR program and gel visualization and analysis were performed in the same manner as in the production of preliminary HBRV construct experiment in the section above. Once it was confirmed full *HBRV su* was produced, the experiment was repeated using the entire quantity of the 200bp and 800bp gel purification products with the ratios between reactants remaining unchanged from the original experiment. The full HBRV template was cloned into Topovector 2.1 (***Life technologies Inc.***) according to the manufacturer's protocol. The full HBRV template was then confirmed using Sanger sequencing performed by The Applied Genomics Center (TAGC) (***University of Alberta***).

2.5 Production of Full *HBRV su* and Truncated HBRV su Constructs

The corrected Full *HBRV su* template was used in conjunction with primers 1, 2, 5 and 6 to produce 4 DNA truncations that will encode the HBRV Su Proteins 1-4 (Figure 2). One master mix was prepared, on ice, for each construct to be amplified. Each included: 5µL of10x Hi FI buffer (***Life technologies Inc.***), 1µL 10mM dNTPs, 2µL 50mM $MgSO_4$ (***Life technologies Inc.***), 5µL of corrected *HBRV su* template, 0.2µL Platinum® Taq High Fidelity Polymerase (***Life technologies Inc.***). A unique combination of two primers at a concentration of 10pmol/µL, 3µL of each primer, in each individual mix was used to produce each construct. From smallest to largest: primers 2 and 5 were used to produce Construct 1, primers 1 and 5 were used to produce Construct 2, primers 2 and 6 were used to produce Construct 3, and primers 1 and 6 were used to produce the Construct 4, which represented the full *HBRV su*. DdH_20 was then added to bring the final volume to 50µL. The PCR program and gel visualization and analysis were performed in the same manner as in the production of preliminary HBRV su DNA truncation experiment in

the section 2.4. Once it was confirmed the HBRV su DNA truncations were produced, the experiment was repeated and the resultant bands were gel purified.

2.6 Inserting HBRV su Constructs into pGEX 4T-2

An enzyme digestion was utilized to insert the HBRV su constructs into the pGEX 4T-2 vector (***GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA***). 5µL of DNA, 0.5µL EcoRI ***(Life technologies Inc., Burlington, ON, Canada)***, 1 µL EcoRI 10x buffer ***(Life technologies Inc.)***, 0.5µL XhoI ***(Life technologies Inc.)***, and 1µL 10x XhoI ***(Life technologies Inc.)*** were mixed together and the final volume was brought up to 10µL with ddH_20. The mixture was incubated at 37°C overnight. Both the pGEX 4T-2 and the HBRV su constructs were digested in this fashion. Subsequent to digestion, the cut pGEX 4T-2 was incubated separately with each HBRV su digestion product and T4 DNA ligase (***Life technologies Inc.***) to yield four final vectors with unique HBRV su inserts. Ligation was done based on the ligase manufacturer's protocol. The various vectors were then gel purified using a QIAquick© Spin Kit (***Qiagen, Missassauga, ON, Canada***) according to the manufacturer's instructions in their microcentrifuge protocol. The products were sequenced by TAGC and then stored at -20°C prior to use.

2.7 Amplification of pGEX 4T-2 Containing HBRV su Constructs

One Shot® Top10 Chemically Competent *E. coli* (***Life technologies Inc., Burlington, ON, Canada***) were transformed with the HBRV su Construct containing pGEX 4T-2 vectors according to the manufacturer's Rapid Transformation Procedure. Each pGEX 4T-2 transformed suspension was then grown on a 100µg/mL ampicillin agar plate for 18 hours at 37°C. All subsequent *E. coli* work utilized media with added ampicillin. Colonies were screened and selected using colony PCR. 10 colonies from each construct's plate and 5 colonies from the plate transformed with the native pGEX 4T-2 vector were selected for PCR. 3µL of primer 2 and 5, chosen because every successful transformed construct would contain template for these primers, were mixed with 5µL 10x buffer, 1µL of 10mM dNTPs, 2µL 50mM MgSO4, 0.2µL enzyme, and ddH_20.

Multiple colonies of each pGEX 4T-2 HBRV su type were selected and grown up in 2mL of LB overnight. A QIAprep® Miniprep (***Qiagen, Missassauga, ON, Canada***) was used according to the manufacturers instructs for plasmid DNA purification using the QIAprep® spin miniprep kit

to recover replicated pGEX 4T-2 vectors from the cells. Plasmid concentrations were tested using a nanodrop 1000 (***Thermo Fischer Scientific, Burlington, ON, Canada***).

The vectors were sequenced by TAGC to ensure presence of correct HBRV su construct sequences. Colonies that successfully produced vectors containing our inserts were grown up overnight and stored at -80°C in 50% glycerol for future vector production.

2.8 HBRV Su Protein Production in *E. coli*

Aliquots of BL21 Competent *E. Coli* (***New England Biolabs Inc., Whitby, ON, Canada***) were transformed with the 4 pGEX 4T-2 vectors, each including a unique HBRV su construct. Transformations followed the manufacturer's protocol, with the exception that LB media was used in place of SOC media. Serial dilutions were also excluded, with 10µL of transformed bacteria being spread on each agar plate instead. BL-21s were then grown on agar plates with 100µg/mL ampicillin, made from ampicillin sodium salt (***Life technologies Inc., Burlington, ON, Canada***), overnight at 37°C. Plates were sealed and stored at 4°C. After protein production and testing, successful colonies were grown up again and stored at -80°C in 50% glycerol. Isolated seeder colonies were then selected using a pipet tip, which was incubated in 2mL of LB with 100µg/mL ampicillin. The seeding culture was grown overnight at 37°C at 200rpm.

Optimal optical density (O.D.) for IPTG introduction, incubation temperature, and harvesting of cells for protein recovery were determined through a series of optimization experiments. O.D. of growing colonies was determined by adding 1mL of the *E. coli* culture to a cuvette to be measured by a spectrophotometer (***Molecular Devices, LLC, Sunnyvale, CA, USA***) at 600nm.

The seeder colony of Inoculated BL-21s was added to 200mL of LB and grown at 37°C in an incubated shaker at 200rpm until O.D. reached 0.6-0.8. O.D. Incubator temperature was dropped to 30°C and protein production was induced by adding ultrapure IPTG (***Life technologies Inc.***) to the medium to a final concentration of 50µM. The *E. coli* were grown in the presence of IPTG until 1.2-1.4 O.D., at which point they were centrifuged at 4°C for 10 minutes at 6000g. The bacteria were re-suspended in ice cold PBS, and spun again for 10 minutes at 6000g to remove traces of media. The pellet was then re-suspended in sonication buffer at a ratio of 2:1, buffer to pellet. The sonication buffer was 50mM Tris-HCL pH 7.5, 100mM NaCl, 1mM DTT, 5%

Glycerol, and 1 Complete, Mini Protease Inhibitor tablet© per 10 mL of solution (***Roche Diagnostics, Mannheim, Germany***).

The buffer/pellet suspension was sonicated using a sonic dismembrator model 100 (***Thermo Fischer Scientific, Burlington, ON, Canada***). Samples were alternated to allow cooling between individual sonication steps with the probe being cleaned with alcohol and distilled water between individual sonications. Sonication occurred at setting 5 three times. Individual sonications lasted 1.5 minutes for 3 seconds on and 6 seconds off. The entire procedure took place on ice in at 4°C. The sample was spun down at 10,000g for 10 minutes at 4°C in an ultracentrifuge, then supernatant and pellet were separated. The pellet was re-suspended in sonication buffer up to the same volume as the supernatant for the purpose of comparing the ratio of target protein in supernatant and in the pellet. Protein was stored at -80°C in small aliquots.

Testing for successful protein production was performed using a small scale GST pull-down. Glutathione Resin (***GenScript USA Inc., Piscataway, NJ, USA***) was prepared by pelleting the beads with a spin for 1 minute at 1000g, then removing the ethanol storage solution and washing with PBS twice. 20µL of glutathione resin was then added to 50-100µL of the GST containing supernatant from control pGEX inoculated cells or 300-500µL of the supernatant containing GST and HBRV Su fusion protein. The proteins were allowed to bind the glutathione resin during a 30 minute incubation at 4°C. The beads were washed 4x with sonication buffer with 0.5% Triton X added. Wash buffer was added and mixed with the beads, then the beads were spun down at 1,000g and the buffer was aspirated. 50µL of 6x SDS loading buffer was then added to the beads, and they were boiled at 100°C for 10 minutes. The beads were spun down at 1000g and the loading buffer was loaded into a 12% SDS gel to test in an SDS-PAGE protocol.

Preliminary evaluation of protein production was performed using SDS-PAGE. The 12% SDS gel's separating gel recipe was 5.3mL ddh_20, 6.4mL 30% Acrylamide, 4mL Tris (1.5M) pH8.8, 160µL 10% SDS, 160µL 10% APS, 16µL TEMED. The stacking gel recipe was: 5.3mL ddH_2O, 2mL 30% Acrylamide, 2.5mL Tris (0.5M) pH 6.8, 100µL 10%SDS, 100µL 10% APS, 10µL TEMED. A 0.75mm 12% SDS gel was prepared. Each protein expressed from its respective construct, and the control GST product, were boiled for 10 minutes in 6x SDS sample loading buffer. The protein was then run on the SDS gel until the 47kD Protein1 band had migrated to the midpoint of the gel. The stacking gel was then trimmed from the separating gel, and the gel

was incubated overnight on a shaker in Coomassie blue staining buffer. SDS destain solution (700 mL ddH_20, 200mL methanol, 100mL glacial acetic acid) was incubated on a shaker for 30 minutes. The destaining step was repeated 3 times before a final step incubating with distilled water. The gel was then visualized using a gel doc (***Bio Rad Laboratories Canada Ltd., Mississauga, ON, Canada***) and analyzed with Quantity One software (***Version 4.6.7, Bio Rad Laboratories Inc.***) to evaluate whether the protein production had been successful.

2.9 GST-HBRV Su Fusion Protein Purification

Large scale purification followed confirmation of successful protein production. 1 mL of Glutathione Resin (***GenScript, Piscataway, NJ, USA)*** was settled in 5 mL gravity purification column. PBS was used to wash the beads, and the column was stored at 4°C overnight to allow removal of air bubbles. GST elution buffer was prepared according to manufacturer's instructions for the Purification of Recombinant GST-Fusion Protein protocol. All steps with the column were completed at 4°C. Supernatant from the lysate produced using the same sonication procedure described in section 2.8 was run over the column three times to maximize yield. The column was then thoroughly washed with ice cold PBS. GST-fusion protein was then eluted drop-wise with GST elution buffer. 3-4 drops were added at a time with 3-4 being collected at a time in tubes. After 6 rounds of drop wise collection, 500µL of elution buffer was added and the flow through was collected in a drop wise fashion. A Bradford assay was utilized to test which fractions contained significant protein for experimentation.

A Bradford Bio-Rad Protein Assay (***Bio Rad Laboratories Canada Ltd., Mississauga, ON, Canada***) microassay procedure for microtiter plates was used to quantify the protein concentrations of the various HBRV Su proteins. Bovine serum albumin (BSA) was selected as the standard for the assay and all samples were analyzed in a flat bottomed 96 well plate. Five dilutions of BSA in GST elution buffer done in duplicate were prepared corresponding to the assay's linear range of 8.0µg/mL to roughly 80µg/mL. 160µL of each standard and sample were combined and mixed, in separate wells, with 40µL of Bradford dye concentrate. The samples were incubated for 15-30 minutes, then absorbance was measured at 595nm in a spectrophotometer. Promising fractions of protein were stored as 5µL aliquots at -80°C for long term storage.

2.10 Standard Antibodies

Purified anti-MMTV gp52 Su and anti-MMTV produced in goats were provided by the lab of Susan Ross (11). The anti-MMTV gp52 Su antibodies (anti-gp52 Su) exhibited antigen specific reactivity for gp52 Su from both MMTV (11) and the antigens produced from HBRV Su expression constructs. The anti-MMTV detects gp52 Su from MMTV and antigens produced from HBRV Su expression constructs, as well as the Transmembrane Env protein gp36 and the viral Gag proteins p14 and p27 (11). Both antibodies were used extensively as standards in Western blot and ELISA work with HBRV antigens.

2.11 HBRV gp52 Su Western Blot (*E. coli*)

The HBRV Su products were tested for antigenicity and to confirm successful production using Western blot. A 1.5mm 12% SDS gel was prepared for the Western blots using antigens boiled for 10 minutes in 6x protein loading buffer. The protein was then run on the SDS gel until the 47kD Protein 1 band had migrated to the midpoint of the gel. Proteins were then transferred to a 0.45µm Nitrocellulose membrane (***Bio Rad Laboratories Canada Ltd., Mississauga, ON, Canada***) at 4°C in western transfer buffer overnight at 80 mA. All subsequent steps were performed at room temperature on a shaker. The membranes were placed in separate containers for further washes and incubations.

The membranes were individually washed with TBST (TBS + 0.05%Tween20) for 5 minutes and cut into individual strips, which were blocked with 5% skim milk in TBST for 15 minutes. The 5% skim milk in TBST solution also acted as diluent for antibodies. Purified polyclonal anti-MMTV were diluted to 1:3000. Monoclonal mouse anti-GST (26H1) (***Cell Signal Technology, Whitby, ON, Canada***) was used according to the manufacturer's specifications. 10mL of these diluted primary antibodies were incubated with the membranes for 1 hour at room temperature. The membranes were then washed three times with TBST for 10 minutes each wash. IRDye® 800CW Donkey Anti-Goat IgG (***LI-COR, Lincoln, NE, USA***) was used as a secondary antibody for anti-MMTV while IRDye® 680LT Goat Anti-Mouse IgG (***LI-COR***) was used as a secondary antibody for anti-GST. The secondary antibody was incubated with the membranes at a concentration of 1:10 000 for 30 minutes. Wells were washed again three times with TBST. Results were then examined using an Odyssey® CLx (***LI-COR***) by reading with the 700 nm

channel for the anti-mouse secondary 800nmchannel for the anti-goat secondary. Results were then analyzed using the manufacturer's default analytical software and Image Studio Version 3.1 (***LI-COR***).

2.12 HBRV gp52 Su ELISA (*E. coli*)

Except where noted, all HBRV Su ELISA (*E. coli*) steps were performed at room temperature (24°C) with all samples and controls performed in triplicate. Nunc Immuno Module Maxisorp plates (***Thermo Fischer Scientific, Burlington, ON, Canada***) were washed twice with 400µL PBS. All subsequent wash steps consisted of one wash with 350µL of PBS with 0.05% Tween20, then two washes with 200µL each. Wash buffer was removed immediately after all wells were filled by inverting the plates, shaking, then striking on paper towels.

Plates were prepared for incubation with serum to block anti- *E. coli* antibodies. Two plates were washed with PBS, then coated with *E. coli* lysate at 1.5ng/µL to a volume of 100 µL/well. These plates were incubated for 18 hours at room temperature. The *E. coli* lysate plates were then washed similar to the gp52 Su plate wash steps. Plates incubated with *E. coli* lysate were not used in the initial plasma screening ELISA, but were used in the later, larger experiment screening PBC and control serum samples.

The next day, two new plates were washed, then coated with 37.5ng of HBRV Su Protein 3 antigen in PBS for 18 hours. Concurrently, the *E. coli* plates were washed with PBST. Then 100µL of each healthy control, diseased control, and PBC patient sera were diluted to 1:300 and were incubated overnight at 4°C on the *E. coli* lysate plates. This step was meant to block any anti-*E. coli* antibodies from the patient serum in order to reduce possible background signal. This incubation was timed to be completed at the same time as the blocking step on the HBRV Su plate.

The HBRV Su plates were washed, then blocked with 400µL of 1% factor V BSA (***Sigma, Saint Louis, MO, USA***) in PBS for 3 hours. The 1% BSA in PBS blocking solution was used in all subsequent dilutions of antibodies. Following blocking, the plates were washed once again. The serum was then transferred from the *E. coli* lysate plate onto the HBRV Su plate and was incubated for 18 hours at 4°C.

100µL of serum was incubated on the plate at 4°C for 20 hours. Plates were washed, then donkey anti-human secondary antibody (***Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA***) was incubated on plates for 3 hours at a concentration of 1:40 000. The plates were washed a final time, then 100µL of room temperature tetramethylbenzidine substrate (TMB) (***Sigma***) was added to each well and was allowed to develop for 20 minutes in darkness. 50uL of 2N H_2SO_4 was then added to each well to stop the reaction. A spectrophotometer (***Molecular Devices, LLC, Sunnyvale, CA, USA)*** and analysis software Softmax Pro 5.4 (***Molecular Devices***) was immediately used to measure the optical density of each well at 450nm. The optical density at 540nm was also measured, and was subsequently subtracted from the 450nm value of the same well to correct for optical imperfections. Results were analyzed using PRISM 4 graphical software ***(Version 4.0b, GraphPad Software, Inc.***).

2.13 Human Betaretrovirus gp52 Su Mammalian Expression Construct

The expression construct for HBRV Su expression in mammalian cells was provided by Dr. Guangzhi Zhang. The construct utilized the pcDNA 3.1 vector (***Life Technologies Inc., Burlington, ON, Canada***) with a clone of the open reading frames of the HBRV signal peptide, the surface protein as well as Polyhistidine (HIS) and FLAG octapeptide purification sequences. A normocin resistance gene was also introduced to the construct. The construct was stably transfected into 293T cells using normocin selection. Following expression of the fusion protein, the HBRV signal peptide targeted the protein to the cellular membrane. The signal peptide was cleaved from the fusion protein consisting of the HBRV gp52 Su and purification sequences, and the fusion protein was secreted into the cell culture media.

2.14 Purification from Stably Transfected 293T Cells Expressing HBRV gp52 Su

The stably transfected cells were grown in media made from high glucose, pyruvate Dulbecco's Modified Eagle Medium (DMEM) (***Life Technologies Inc., Burlington, ON, Canada***) with 10% FBS (***Life Technologies Inc.***), 0.1 mg/mL normocin (***InvivoGen, San Diego, CA, USA***). In all further cell culture work, this mixture was used as growth media, unless otherwise specified. Stably transfected 293Ts were grown to 80% confluence, or roughly $7.4x10^6$ cells, on 100mm tissue culture plates (***Sarstedt, Montreal, QC, Canada***). These cells were then removed from the plate using a cell scraper in presence of media and were gently pelleted via centrifuge. The

media was aspirated, then the cells were suspended in freezing media made from 50% media, 40% FBS, and 10% DMSO. The 1 mL aliquots were stored in liquid nitrogen. For protein expression work, cells were thawed at room temperature added to growth media and plated on 100mm plates.

Stably transfected HBRV gp52 Su producing 293Ts were grown up on 100 mm tissue culture plates until confluent. They were then split onto 150mm tissue culture dishes (***VD Falcon, Mississauga, ON, Canada***) and grown in 25mL of media on each plate before being split again upon confluence. When cells were confluent, serum free media (***Lonza, Allendale, NJ, USA***) was introduced and was harvested 48 hours later. New serum free media was then introduced and harvested after a further 48 hours. For the first purification attempts, the 48 hour medium was stored at 4°C until the 96 hour medium was collected. During later protein production efforts, purification immediately followed media collection.

Next the 300mL of total media was purified using an ÄKTAexplorer 100 air, a device for performing fast protein liquid chromatography (FPLC), and a HisTrap HP 1mL column (***GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada***). Buffers, including the binding and elution buffers that were also used in later steps, were prepared according to the HisTrap HP manufacturer's protocol. Protein storage buffer was composed of 10% glycerol, 100 mM KCl, 50mM Tris pH7.7, with 1mM DTT added before use. The ÄKTAexplorer was used to purify the HIS-tagged HBRV Su through a fast protein liquid chromatography protocol in which the protein containing media was first run through the HisTrap HP column. The column was washed with binding buffer containing low concentration imidazole to reduce non-target protein contamination. Elution took place using elution buffer in a single step ranging from 0% to 100% elution buffer with protein fractioned in 1mL aliquots. Aliquots were eluted into 0.5mL of storage buffer. In the second round of purification, both 250mL collections of media were purified immediately upon recovery. Purified protein samples were stored at -80°C.

Following purification, a Pierce™ BCA Protein Assay Kit (***Thermo Scientific, Rockford, IL, USA***) microplate procedure was utilized to determine the protein concentrations of each purification fraction the FPLC's spectrophotometer had suggested contained a significant concentration of protein. BSA was diluted into 9 different concentrations from 2000µg/mL to 0µg/mL in 2/3 elution buffer and 1/3 protein storage buffer from the purification procedure.

Protein samples were thawed, then 25µL of each sample and standard were pipetted into a flat-bottomed 96 well plate in triplicate. 200µL of working reagent was added to each well and mixed with the samples by gently pipetting up and down 15 times. The plate was incubated for 30 minutes at 37°C before absorbance was read on a spectrophotometer at 562nm.

In preparation for the HBRV gp52 Su ELISA Version 3 and HBRV Su Western blots, Dr. Guangzhi Zhang expressed and purified HBRV Su, which was then provided as a gift. Stably transfected 293Ts were grown up and media was collected in a similar manner to the previous purification protocol. However, purification did not utilize FPLC. Media was pumped through a HisTrap FF crude column (***GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada***). Purification took place at 4°C, with purified antigen being subject to buffer exchange in order for it to be stored in 100% storage buffer. Protein concentration was confirmed via BCA assay.

2.15 HBRV gp52 Su ELISA Version 1

Except where noted, all ELISA steps were performed at room temperature with all samples and controls run in duplicate. Optimal concentrations for 1° antibodies, 2° antibodies, and antigen, as well as durations of incubations, were ascertained through a series of optimization experiments. Nunc Immuno Module Maxisorp plates from Fisher Scientific were washed twice with 380µL PBS. All subsequent wash steps consisted of 5 individual washes with 200µL of PBS with 0.05% Tween20. Each individual wash was incubated on the plates for 1 minute. Wash buffer was removed by inverting the plate, then striking repeatedly on paper towel. Plates were then coated with 100µL of 2ng/µL gp52 Su antigen in PBS for 18 hours. Plates were washed, then blocked with 380µL of 1% BSA (***Sigma, Saint Louis, MO, USA)*** in PBS for 3 hours. The 1% BSA in PBS blocking solution was used in all subsequent dilutions of antibodies. All serum samples used in the ELISA were exposed to two freeze thaw cycles. Following blocking, the plates were washed once again; however, in the final wash, the wash buffer was incubated on the plates for 1 hour at 4°C. This incubation allowed time for the PDC-E2 blocking step to take place.

Healthy control and PBC patient sera were diluted to 1:400, and were then incubated for 20 minutes at 4°C with 2n/µL PDC-E2 (***Sigma***) to block AMA antibodies in PBC sera. Concurrently, anti-gp52 Su and anti-MMTV antibodies were diluted to 1:40 000 and incubated with PDC-E2 in the same manner as the serum samples. Anti-gp52 Su and anti-MMTV

antibodies were used as positive antibody controls as both exhibit reactivity to gp52 Su. 100µL of serum and positive antibody controls were incubated on the plate at 4°C for 20 hours. Plates were washed, then secondary antibody was incubated on the plates for 3 hours at a concentration of 1:30 000. For anti-gp52 Su and anti-MMTV, donkey anti-goat secondary antibody (***Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA***) was used while donkey anti-human antibody was employed for the human serum. The plates were washed a final time, then 100µL of room temperature tetramethylbenzidine substrate (TMB) (***Sigma***) was added to each well and allowed to develop for 20 minutes. 50uL of 2N H_2SO_4 was then added to each well to stop the reaction. A spectrophotometer was used to measure the optical density of each well at 450nm. The optical density at 540nm was also measured, and was subsequently subtracted from the 450nm value of the same well to correct for optical imperfections. Results were analyzed using PRISM graphical software (***GraphPad Software, Inc)***.

2.16 HBRV gp52 Su ELISA Version 3

Except where noted, ELISA steps were performed in accordance with the protocol in section 2.15. Experimental conditions were modulated from the HBRV gp52 Su ELISA version 1 in an attempt to replicate the conditions used by InBios, a biotech company specializing in serological diagnostics, in version 2 of the ELISA. Microlon flat bottomed high binding plates from Greiner bio-one (***Greiner, Monroe, NC, USA***) were used as plates. Wash steps were changed, and consisted of 6 individual washes, the first with 300µL PBS with 1% Tween20, and the rest with 200µL. There was no PDC-E2 blocking step.

During the 3 hour block, all serum and primary antibody dilutions were prepared then transferred onto a 96 well plate in preparation for use in the ELISA. Instead of a single standard antibody concentration, anti-gp52 Su was diluted in a standard curve from 1:1000 to 1:128 000 on each plate.

100µL of serum and positive antibody controls were incubated on the plate at 37°C for 1 hour. Following washing, the secondary antibody was incubated on the plates for 1 hour at 37°C at a concentration of 1:30 000. Room temperature tetramethylbenzidine substrate (TMB) (***Sigma, Saint Louis, MO, USA***) was allowed to develop for 38 minutes, as opposed to the 20 minutes used in previous ELISA work. The anti-gp52 Su standard curve was used to standardize results

between plates and combine the data to one set. Results were analyzed using PRISM graphical software (***GraphPad Software, Inc)***.

2.17 HBRV gp52 Su Western Blot (Mammalian)

A 1.5mm 10% SDS gel was prepared for a Western blot. 2µg of gp52 Su was boiled for 10 minutes at 100°C in 17.24µL of 2x loading buffer, with the total volume being brought up to 200µL with 1x protein loading buffer. 2x loading buffer consisted of 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 100mM Tris-Cl (pH6.8). 1x loading buffer was produced by diluting the 2x loading buffer 1:1 in ddH_20. The protein was then run on the SDS gel at 80V until the protein entered the separating gel. At this point, the gel was run at 140V until the 50kD ladder band had migrated to the midpoint of the gel.

Protein was then transferred to a 0.45µm Immobilon-P PVDF membrane (***Millipore, Bedford, MA, USA***), which was pre-wet with methanol at room temperature for one minute. The protein transfer took place at 4°C in cooled western transfer buffer. Protein was transferred at 90V for 45 minutes. All subsequent steps were performed at room temperature on a shaker. The PVDF membrane was cut into up to 16 strips, which were then placed in separate wells for further washes and incubations.

The membrane was washed with 600µL of PBST (PBS + 0.05%Tween20) for 5 minutes. Strips were subsequently cut from the membrane and blocked with Odyssey® Blocking Buffer (***LI-COR, Lincoln, NE, USA***) for 30 minutes. Odyssey® Blocking Buffer was used as diluent in serum and primary antibody dilution steps. Serum was diluted to 1:20 and 1:50 while the antibody positive control anti-gp52 Su was diluted to 1:10 000.

600µL of these primary antibodies were incubated with the strips for 1 hour at room temperature. Following the incubation, the strips and wells were washed with distilled water, then the strips were washed three times with 600µL PBST for 10 minutes each wash. IRDye® 800CW Goat Anti-Human IgG (***LI-COR***) was used as a secondary antibody for serum wells and IRDye® 800CW Donkey Anti-Goat IgG (***LI-COR***) was used as a secondary antibody for anti-gp52 Su. The secondary antibodies were incubated with their prospective targets at a concentration of 1:15 000 for 30 minutes. Following the incubation, the strips and wells were washed with distilled water, and the wells were washed again three times with 600µL PBST before being washed a

final time with 600µL of distilled water. Results are then examined using an Odyssey® CLx (***LI-COR***) by reading at 800nm. Results were then analyzed using the manufacturer's default analytical software and Image Studio Version 3.1 (***LI-COR***).

Chapter 3: Results

3.1 Human Betaretrovirus su Truncations from *E. coli*

Overlapping 200 and 800bp HBRV su truncations were cloned from the Mason lab's stored HBRV *env* with two sets of distinct primers. The purpose of these truncations was to correct a single nucleotide polymorphism in our lab's *HBRV su* compared to the published *HBRV su* sequence. Visualization confirmed successful production of the truncations. Template contamination was noted in the controls as much fainter bands at 200 and 800bp next to their respective experimental lanes. However, as no incorrectly sized bands were present, the 200 and 800bp HBRV su truncations were subsequently used to produce corrected full *HBRV su* template.

A 1:1 ratio of the gel purified 200bp and 800bp produced the most focused band, and the PCR produced full length *HBRV su* template was confirmed via sequencing. Constructs 1-4 were successfully cloned and verified by detection on an agarose gel. Individual bands were gel purified, processed by enzymatic digestion and cloned into the expression vector pGEX 4T-2. Therefore, four constructs composed of pGEX 4T-2 with HBRV su inserts of varying length were produced. BL21 *E. coli* were transformed with the four constructs. Minipreps of plasmids from positive colonies were sequenced to confirm accuracy and were stored for the expression studies.

3.2 HBRV gp52 Su Protein Production in *E. coli*

Initial expression of protein from transformed *E. coli* at 37°C and 100μM IPTG resulted in successful protein production, but following sonication the vast majority of protein was present in the bacterial pellet and not the soluble fraction. Following optimization, the best results were achieved using 50μM IPTG at 30°C. Control Glutathione S-Tranferase (GST) and two partial HBRV su truncations were clearly produced successfully. Protein 2 expression failed, despite numerous attempts, even though presence of construct 2 in the pGEX 4T-2 vector was confirmed in the BL21s. The sizes of the expressed proteins were verified via Western blot, and were as expected: GST- 30kD, Protein 1- 47kD, and Protein 3- 61kD. It was initially unclear whether Protein 4 had been expressed. In a 12% gel, the band produced by Protein 3 was indistinguishable in size from protein expressed by cells transfected with Protein 4. While the proper expression of Protein 4 was not confirmed, it was used in subsequent ELISA optimizations as a candidate antigen. Subsequent results of the ELISA utilizing anti-MMTV gp52 Su suggested that Protein 4 expression was successful.

The expressed proteins were present in the soluble fraction following sonication. The concentrations of eluted purified proteins were: GST- 24mg/mL, Protein 1- 1.52 mg/mL, Protein 3- 2.57 mg/mL, Protein 4- 2.48 mg/mL. SDS-PAGE using Coomassie blue staining exhibited non-target protein banding present in the protein product even after purification.

3.3 HBRV gp52 Su Western Blot (*E. coli*)

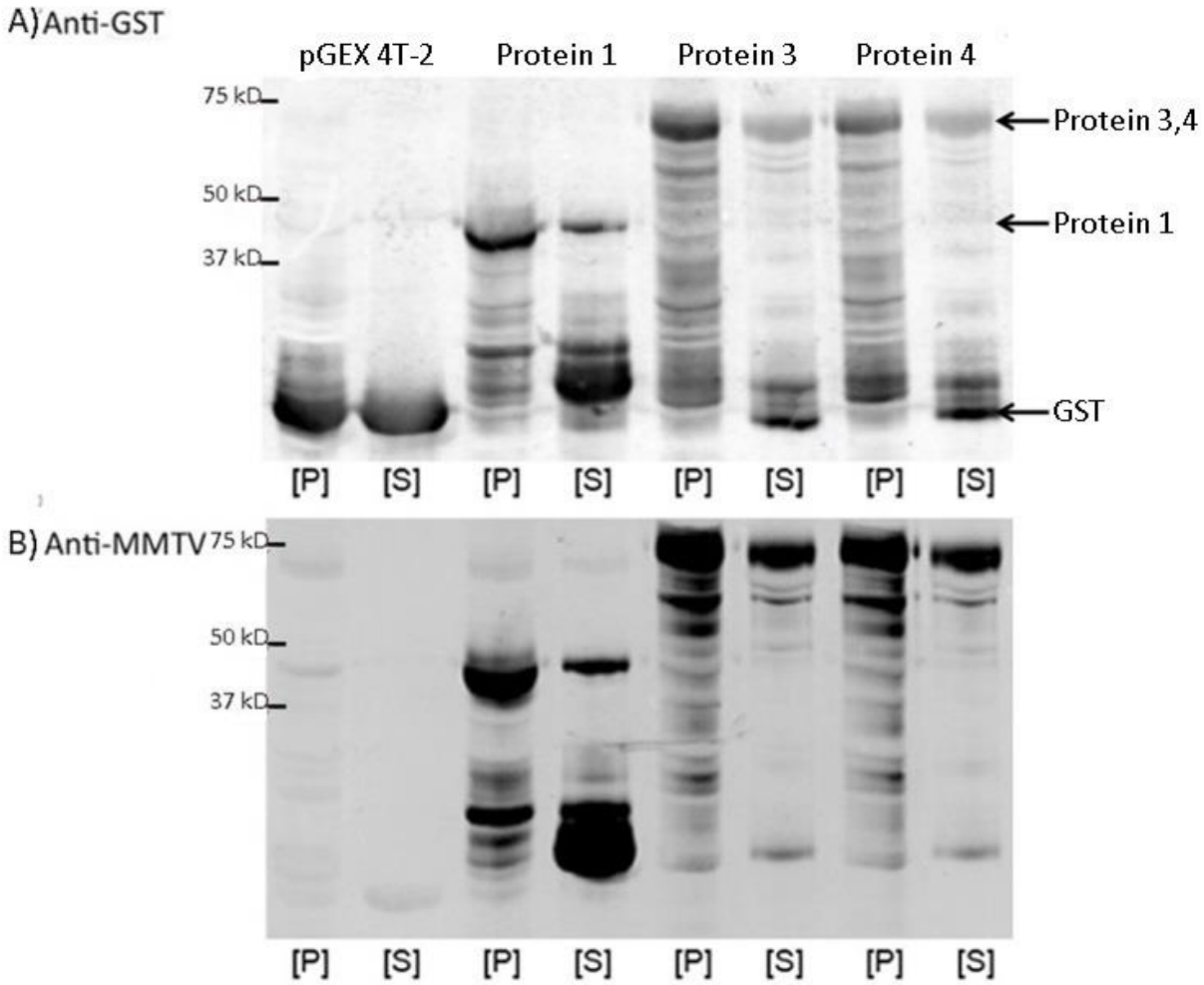
Western blots were used to verify that protein collected from *E. coli* lysates were the expected protein products from each expressed construct. Anti-GST and anti-MMTV antibodies were utilized to insure that antigenic protein products had successfully been expressed with the expected molecular weight.

Using the full-length HBRV *env* gene product as a reference, the Protein 1 protein included amino acids 182-335, Protein 2 amino acids 103-335, Protein 3 amino acids 182-455, and Protein 4 amino acids 103-455. As a result, the GST fusion protein sizes were calculated to be 47.3kD for Protein 1, 56.1kD for Protein 2, 61.1kD for Protein 3, and 69.9kD for Protein 4.

Figure 3:

Western Blot of HBRV Su Expressed in *E. coli*.

The expression and antigenicity of Proteins 1, 3, and 4 were confirmed by Western blot. Expressed HBRV Su proteins were run in a SDS-PAGE gel, transferred to PVDF membrane, and probed with antibodies. (A) Anti-GST antibody was used to confirm protein size, retention of GST-tag to HBRV Su antigen, and confirm successful production of control GST from native pGEX 4T-2 vector. GST also assisted in highlighting degradation products. (B) Anti-MMTV antibody confirmed expression and antigenicity of expressed proteins [P, pellet and S, supernatant].



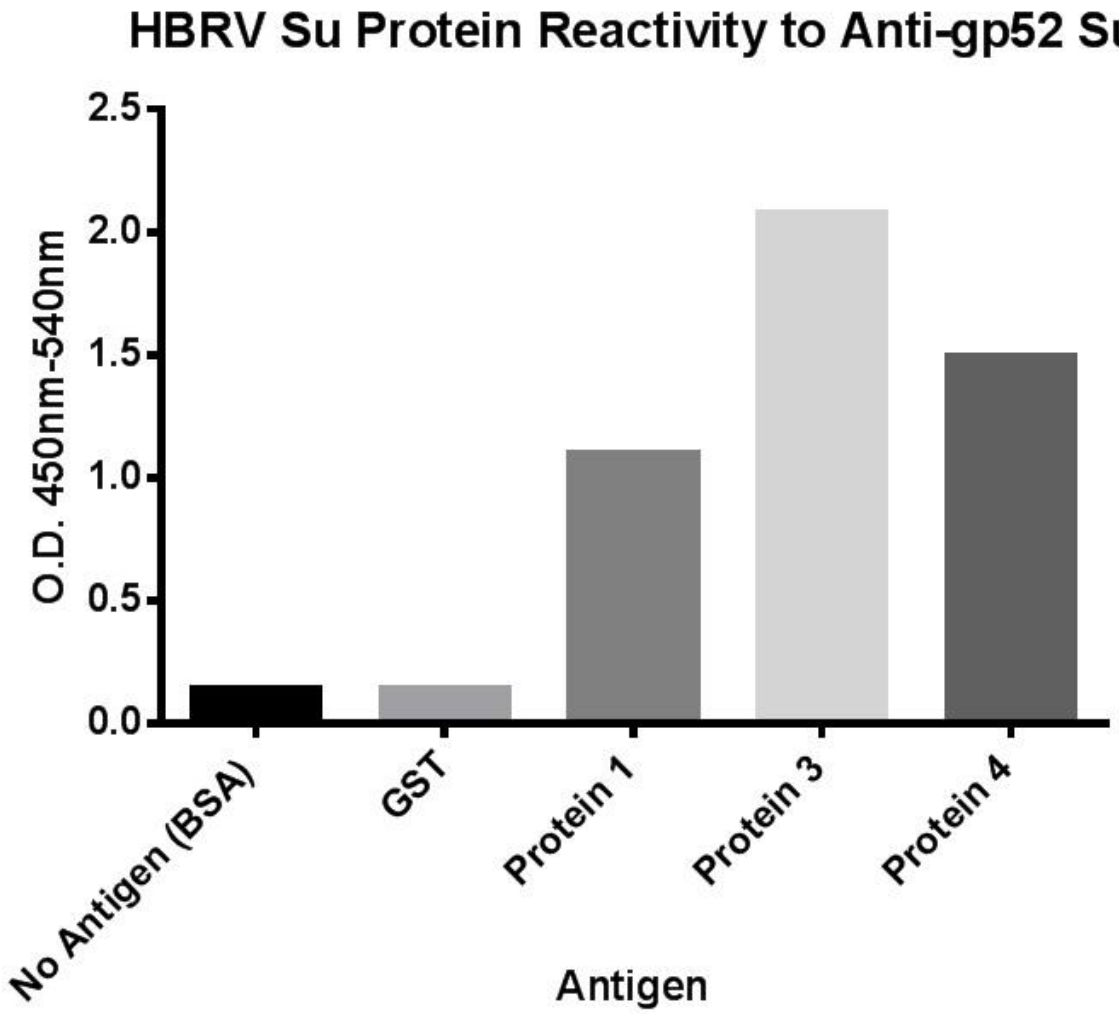
From these studies, Protein 1 and 3 fusion proteins were considered to be successfully expressed antigens, whereas Protein 4 was indeterminate but was carried forward as a candidate antigen for ELISA (Figure 3). Efforts to produce Protein 2 failed despite repeated attempts. GST from bacteria inoculated with native pGEX 4T-2 was also produced successfully and was present as a

30kD band by Western blot. SDS- PAGE exhibited that significant quantities of contaminant proteins were present following purification of HBRV Su antigens. Furthermore, by Western blot, the purification product contained large quantities of GST containing degradation products. Degradation was especially apparent for Protein 1. The presence of protein contaminants and extensive degradation products suggested that purification efforts did not successfully isolate the desired HBRV Su proteins.

Figure 4:

HBRV Su Protein Reactivity to Anti-gp52 Su

HBRV Su proteins were plated in 96 well plates before being blocked with BSA, then incubated with anti-MMTV gp52 Su antibody, then a secondary anti-species HRP-conjugated antibody and followed by TMB substrate. A spectrophotometer was used to measure absorption of wells. Protein 3 exhibited the strongest reactivity to anti-gp52 Su, and was used for subsequent ELISA studies [O.D., optical density].



3.4 HBRV gp52 Su ELISA (*E. coli*)

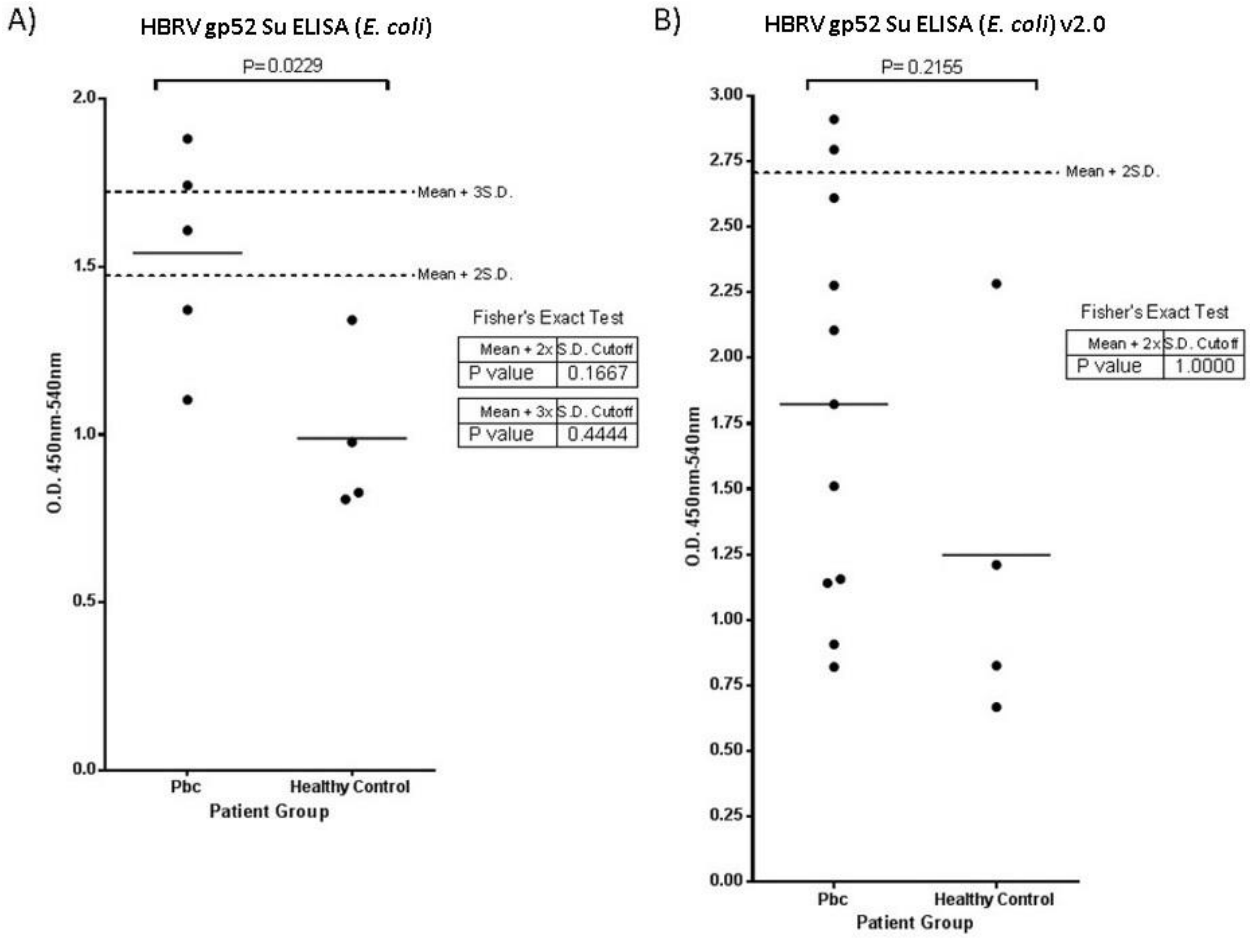
Preliminary ELISA results using anti-MMTV gp52 Su antibodies showed little reactivity to BSA and to GST, suggesting the GST portion of the fusion protein contributed no background reactivity (Figure 4). Protein 3, Protein 4, and Protein 1, listed in descending order of reactivity, all showed significantly higher reactivity to the anti-MMTV gp52 Su antibody than observed with the GST protein. Therefore the initial assay was validated against anti-MMTV gp52 Su antibodies. As Protein 3 had displayed the greatest reactivity to anti-MMTV gp52 Su, it was selected as the antigen of choice for ELISAs probing human samples.

A colleague's T cell epitope study suggested that 45% of PBC patients' peripheral blood mononuclear cells produced interferon-γ and tumor necrosis factor-α following stimulation with HBRV Gag peptides suggesting prior exposure to HBRV infection. Preliminary case control studies utilizing these reactive patients' plasma were undertaken utilizing Protein 3. Significance was determined using two cutoffs: control mean+2x standard deviation (mean+2x S.D.) and control mean+3x standard deviation (mean+3x S.D.). This study showed higher mean reactivity of PBC patients versus healthy controls in this select group (Figure 5). Using the mean+3x S.D. cutoff, 3/5 (60%) of PBC patients were found to be positive versus 0/4 controls. When studying a larger group of PBC patients no samples exceeded the mean+3x S.D. cutoff, however, and only 2/11 PBC patients and 0/4 controls exceeded the mean+ 2x S.D. cutoff. Further optimizations failed to improve discrimination between groups. ELISA studies utilizing HBRV gp52 Su proteins produced in *E. coli* were abandoned in favour of gp52 Su produced from mammalian cells.

Figure 5:

HBRV gp52 Su ELISAs (*E. coli*)

A 96 well plate was coated with Protein 3 HBRV Su antigen before blocking and subsequent exposure to sera from PBC patients and healthy controls. Following binding of serum antibodies, secondary anti-species HRP-conjugated antibody was incubated in the wells. Finally, TMB substrate was incubated in each well. A spectrophotometer was used to measure the optical density [O.D.] of 450nm-540nm absorption of wells. Mean reactivity between PBC patients and controls were examined by two-tailed parametric T-test. Two cutoffs for positivity were set at mean+2x S.D. and mean+3x S.D., and positive samples were compared between groups using Fisher's exact test. Statistical tests and scatterplots were prepared using Prism Graphpad software. A) HBRV gp52 Su ELISA (*E. coli*) using 5 select PBC patients and 4 healthy controls [T test, P=0.023, Fisher's exact test for mean+2x SD cutoff, P=0.17]. B) HBRV gp52 Su ELISA (*E. coli*) v2.0. 11 PBC patients and 4 healthy controls [T test, P=0.22, Fisher's exact test for mean+2x SD cutoff, P=1].



3.5 Expression and Purification of HBRV gp52 Su from Stably Transfected 293T Cells

The FPLC purification using the HisTrap column was utilized to concentrate the HBRV gp52 Su produced in the stably transfected 293T cells. Aliquots of protein eluted by FPLC were tested for protein concentration via a BCA assay (***Thermo Scientific, Rockford, IL, USA***). Protein was found to be concentrated in the first 4 aliquots eluted from the HIS-tag column. Measured aliquot protein concentrations for three distinct purifications of HBRV gp52 Su containing media were between 0.173 mg/mL and 0.617 mg/mL, with the higher concentrations being found in earlier elution fractions released by the FPLC. Following quantification, all protein solutions were stored at -80°C for use in ELISA and Western blot experiments.

The purified HBRV gp52 Su was analyzed by Western blot to quantify and assess antigenicity of the product. A strong band was noted at 52kD in SDS-PAGE using Coomassie blue staining, suggesting the gp52 Su was successfully produced and concentrated. However, the protein product contained a second band of larger sized protein of unknown identity. This additional product was present as a markedly fainter band on SDS-PAGE (Figure 6A), suggesting low concentration, and was absent on Western blot (Figure 6B), suggesting it was unlikely to be a dimer of gp52 Su. As such, the mammalian expressed HBRV gp52 Su was used as antigen for further serological studies. Western blot results utilizing anti-MMTV gp52 Su antibody suggested that the HBRV gp52 Su was produced, and that there was not significant degradation of the protein product.

The antigen produced for the HBRV gp52 Su Western and ELISA version 3 was produced by Dr. Guangzhi Zhang. The protein utilized for the ELISA version 3 had a concentration of 0.566 mg/mL while that used for the Western blots was 0.116 mg/mL. The expressed protein was also exposed to more freeze thaw cycles than the HBRV gp52 Su produced for the HBRV gp52 Su ELISA versions 1 and 2. The antigen was stored at -80°C until use in ELISA and Western blot experiments.

3.6 HBRV gp52 Su ELISA Version 1

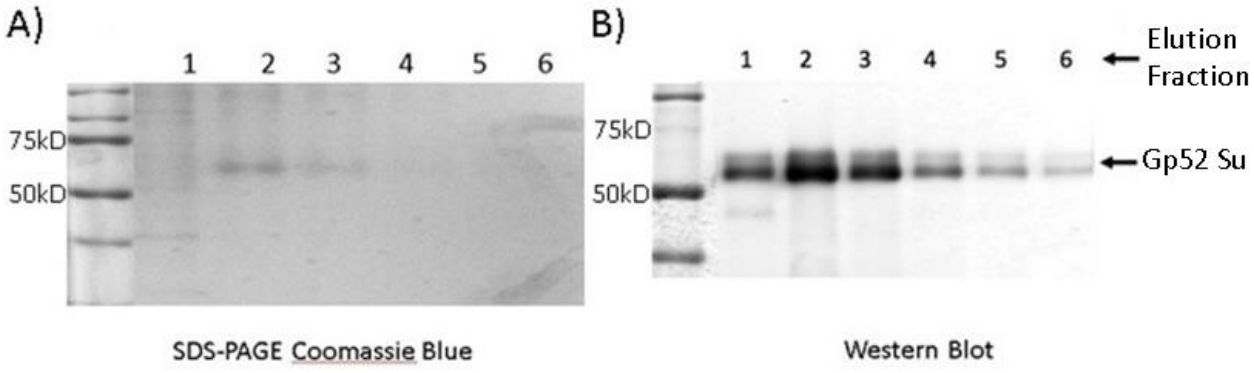
The HBRV gp52 Su ELISA tested whether PBC patient sera samples exhibited greater humoral responses to the HBRV than healthy control sera. Quantities of antigen, and concentrations of

serum and secondary antibody to be used in the ELISA were determined by optimization using anti-MMTV gp52 Su and select patient samples.

Figure 6:

HBRV Su (Mammalian) Purification Product.

Media was collected after incubation on 293T cells stably transfected with an expression vector containing *HBRV su*. A HIS-tag column was used to collect secreted HBRV gp52 Su, which was then purified using FPLC. Individual fractions of protein were run on SDS page. A) SDS-Page with Coomassie blue staining showing HBRV gp52 Su with some presence of minimal large molecular weight contaminants. B) Western blot showing purified HBRV gp52 Su reactivity to anti-MMTV gp52 Su antibody.



Six patient samples were included as controls in the ELISA, but were removed from analysis over concerns they represented poor healthy controls for establishing baseline reactivity for calculating the cutoff values. One patient with high HBRV reactivity was removed due to having an unknown bleeding disorder, which could be a consequence of underlying liver disease. The remaining five removed patients were lab technicians.

The data on three plates from two experiments testing 71 PBC patients and 44 healthy controls were pooled for analysis. The plates were completed in an identical manner to an optimized ELISA protocol, and had no discernable differences in experimental technique. To adjust for variations between plates, the values of all plate samples were normalized to the anti-MMTV gp52 Su positive control readings on the plate. O.D. readings of each well were divided by the anti-MMTV gp52 Su reading on the plate to provide a ratio that was pooled with the remaining readings from the 3 plates and used for statistical analysis.

A T-test of the ELISA results revealed that the PBC samples showed higher reactivity to HBRV gp52 Su than controls (P=0.0008). Using the mean+2x S.D. cutoff, 14/71 (19.7%) of the PBC

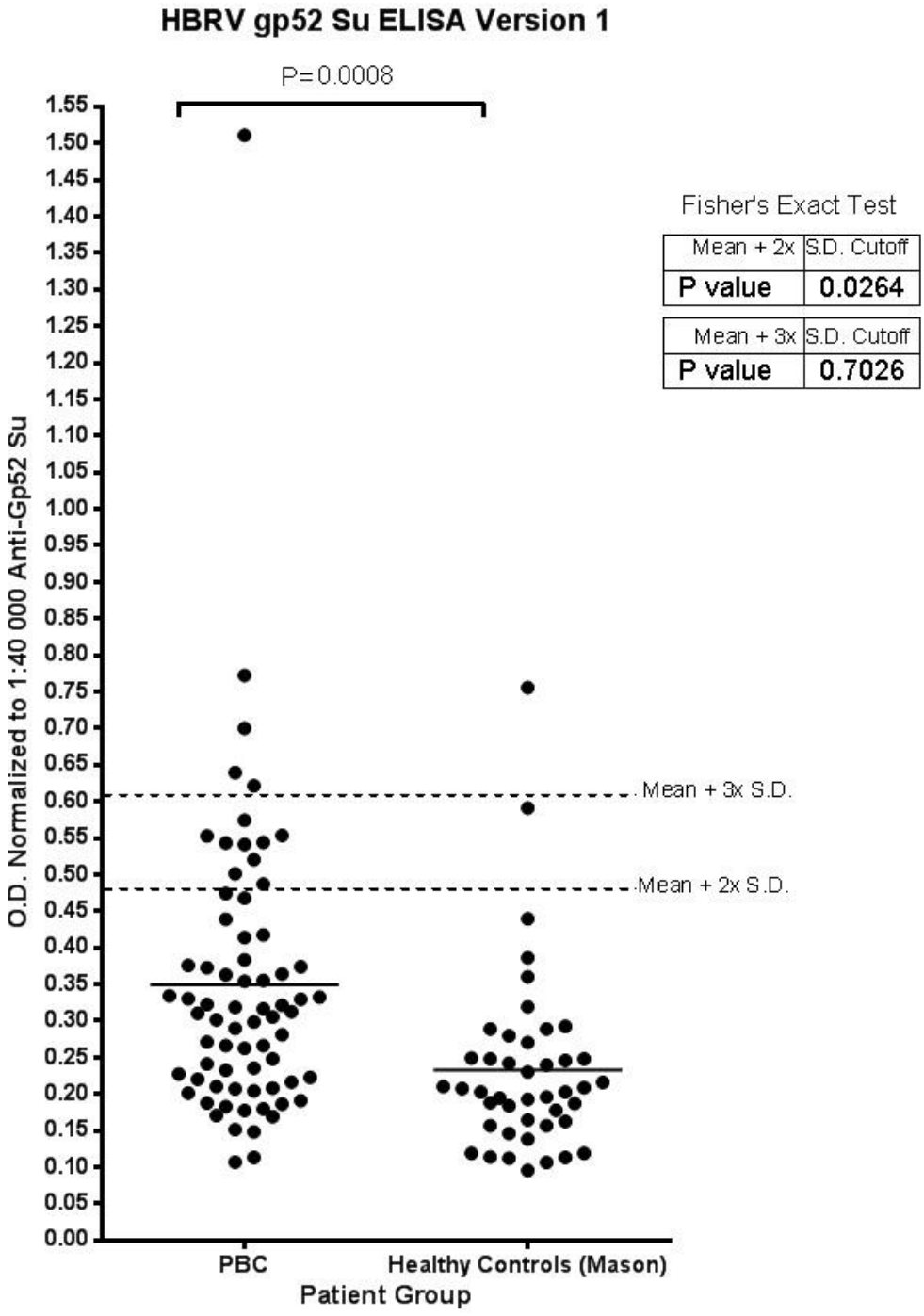
patients and 2/44 (4.5%) of the healthy controls were considered positive (Fisher's exact test, P=0.026), whereas a mean+3x S.D. cutoff suggested that only 5/71 (7%) of PBC patients and 1/44 (2.3%) of healthy controls were positives (Fisher's exact test, P=0.7). The coefficient of variance (CV) of the analyzed data was 5.65% ± 6.76%, 95% CI (4.46%, 6.85%).

These preliminary results suggested that by the less stringent threshold of mean+2x S.D., PBC patients generally exhibited higher reactivity to gp52 Su than did healthy controls.

Figure 7:

HBRV gp52 Su ELISA Version 1.

The results from 3 distinct ELISAs sampling 71 PBC patients and 44 healthy controls were pooled for analysis with results standardized by using a ratio of sample optical density [O.D.] to each plate's control well of 1:40 000 anti-MMTV gp52 Su [T test, P=0.0008, Fisher's exact test for mean+2x SD cutoff, P=0.026, CV 5.65% ± 6.76%, 95% CI (4.46%, 6.85%)].



Five lab technicians and one patient with an unknown bleeding disorder were removed from the above analysis as they represented inappropriate healthy controls with possible exposure to betaretrovirus infection for the former and unknown pathology for the latter. When these data points were included in analysis, PBC patients did not exhibit higher mean reactivity to gp52 the healthy controls (T-test, P=0.092). 1/71 of the PBC patients and 1/50 of the healthy controls were positive by the mean+2x S.D. cutoff (Fisher's exact test, P=1). Using the mean+3x S.D. cutoff produced identical results.

3.7 HBRV gp52 Su ELISA Version 2

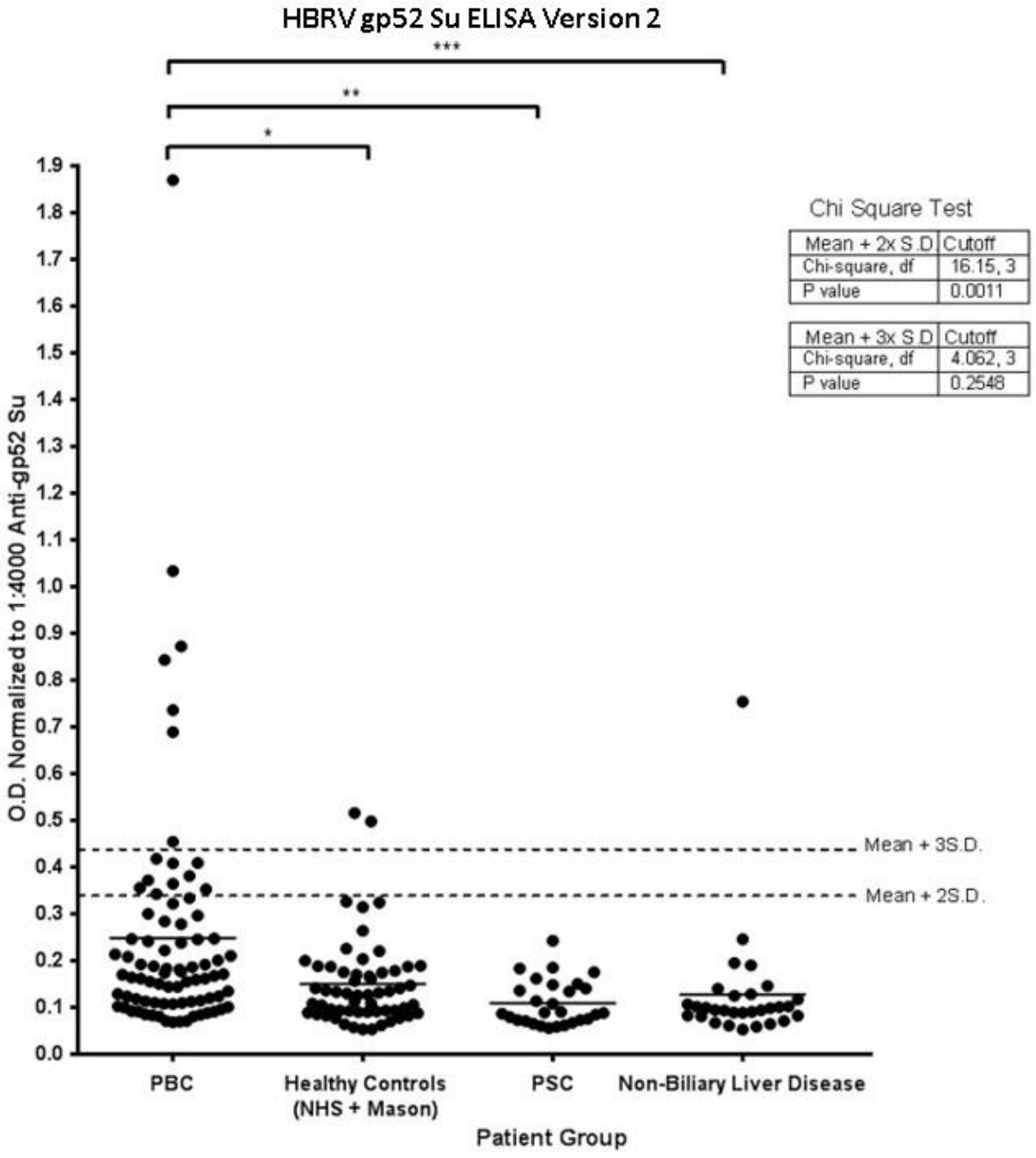
The developed ELISA protocol along with serum samples, anti-MMTV gp52 Su, anti-MMTV, and mammalian expressed HBRV gp52 Su were shipped to InBios, a Seattle based biotech company collaborating to establish a commercial ELISA to diagnose antibody reactivity to HBRV. In Bios examined sera from PBC patients, two panels of healthy controls, and a variety of liver disease control patients against HBRV gp52 Su. InBios tested PDC-E2 blocking and found it to be unnecessary as the blocking step did not significantly impact the test results.

In addition to the healthy controls provided by the Mason Lab, InBios contributed their own normal healthy subjects as another comparison group to PBC patients. In total the samples were derived from PBC patients, biliary disease patients with primary sclerosing cholangitis, and non-biliary liver disease patients with non-alcoholic steatohepatitis (NASH) or hepatitis, healthy subjects derived from the Mason lab and from Seattle. The results from the HBRV gp52 Su ELISA version 2 showed that PBC samples had a higher mean reactivity to HBRV gp52 Su than healthy controls, PSC patients, and non-biliary liver disease patients (T-test, P=0.001, P=0.006 and P=0.016, respectively). Using the mean+2x S.D. cutoff, 16/82 (19.5%) of PBC patients, 0/28 (0%) of the PSC patients and 1/30 (3.3%) of the non-biliary liver disease patients and 2/58 (3.4%) healthy controls were positive (Chi-square, P=0.0011). Using mean+3x S.D. cutoff found 7/82 (7.8%) PBC patients were positive and identical results were observed to the previous cutoff for the non-PBC groups (Chi-square, P=0.2548). Of note, the one patient who was positive in the non-biliary controls suffered from breast cancer.

Figure 8:

HBRV gp52 Su ELISA Version 2.

Serum from PBC patients (n=82), biliary disease patients with primary sclerosing cholangitis (n=28), and non-biliary liver disease patients with NASH or hepatitis (n=30), healthy subjects (n=58) derived from the Mason lab and from Seattle. The results were pooled and standardized by taking a ratio of sample optical density [O.D.] to each plate's control well of 1:4000 anti-MMTV gp52 Su. [T test, * PBC vs healthy controls, P=0.0013, ** PBC vs PSC, P=0.006 and *** PBC vs. non-biliary disease P=0.016, Chi-square all groups using mean+2x SD, P=0.001].



Five lab technician and one bleeding patient were removed from the above analysis due to representing poor healthy controls. When these data points were included in analysis, PBC patients still exhibited higher mean reactivity to gp52 the healthy controls (T-test, P=0.031). However, using the mean+2x S.D. cutoff, 6/82 of the PBC patients, 3/64 of the healthy control, 0/28 NASH patients, and 1/30 non-biliary liver disease patients were positive (Chi-square, P=0.472). Using the mean+3x S.D. cutoff found 1/64 healthy controls to be positive, with the remaining group findings identical to the previous cutoff (Chi-square, P=0.201).

3.8 HBRV gp52 Su ELISA Version 3

The protocol for a final ELISA using a larger sample size was put together using the HBRV gp52 Su ELISA version 1 protocol adopting some of the changes from the ELISA version 2. However, the nature of some of the reagents utilized by InBios in the ELISA version 2 was not disclosed due to propriety issues. A larger sample size was utilized to provide greater statistical power, and a more representative group of healthy controls consisting of healthy middle-aged women whose samples had been previously utilized as controls in a breast cancer study. The latter were considered a better control group as ~ 90% of patients with PBC are women and most are middle aged.

Three duplicate plates of samples comprising 103 PBC patients and 102 controls were pooled for analysis. Standard curves of anti-MMTV gp52 Su ranging from 1:2000 to 1:128 000 were used to standardize between plates. One plate was chosen as the master plate with the standard curves of the other two plates being graphed against that of the master plate. This produced a curve that could be used to standardize the values from the experimental plate to the master plate. The polynomial standard curves produced from graphing the standard curves of the plates against the master plate were good fits with both R^2 values >0.999.

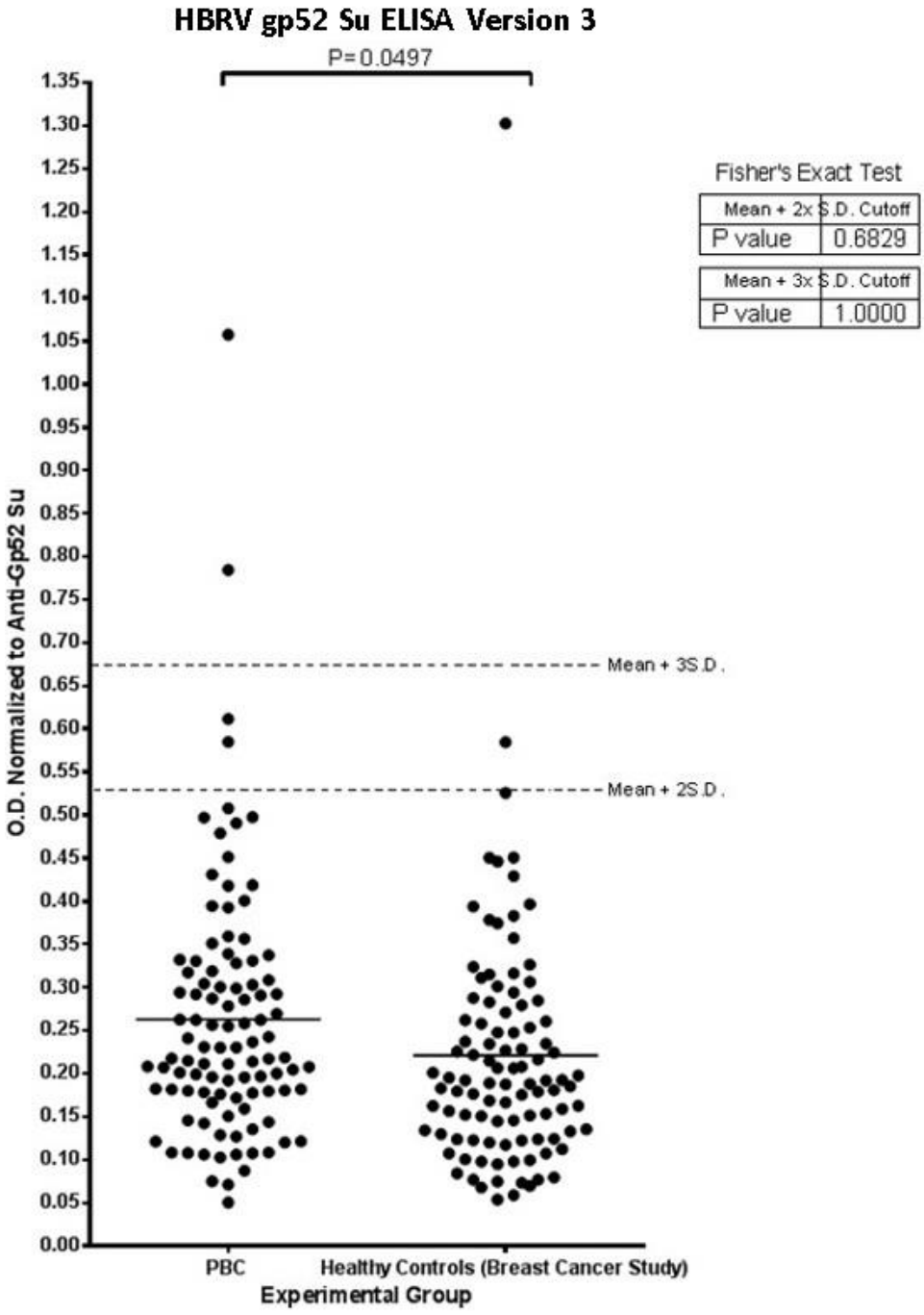
Following standardization of the results, the mean HBRV reactivity was observed to be higher in PBC patients as compared to the female controls (unpaired T–Test, P= 0.0497). Using the mean+2x S.D. cutoff, 4/103 (3.9%) of the PBC patients and 2/102 (2%) of the healthy controls were positive, whereas using mean+3x S.D. cutoff only 2/103 (1.9%) of PBC patients and 1/102 (1%) of health controls were considered positive (Fisher's exact test, P=0.6829 and P=1.000 respectively). The CV of the analyzed data was 8.74% ± 8.39%, 95% CI (7.59%, 9.90%).The

results of the study suggested that under the conditions of this ELISA, only a small minority of patients could be considered positive and there was no association observed between reactivity to HBRV gp52 Su and patient group.

Figure 9:

HBRV gp52 Su ELISA Version 3.

The sample size included 103 PBC patients and 102 healthy breast cancer study control samples pooled from 3 experiments. The results were standardized using serial dilutions of anti-MMTV gp52 Su. Although a higher mean value was observed in PBC patients (T-test, $P<0.05$), the proportion of positive tests was low and showed no significant difference between the PBC patients and controls [CV 8.74% ± 8.39%, 95% CI (7.59%, 9.90%),O.D. optical density].



3.9 HBRV gp52 Su Westerns (Mammalian)

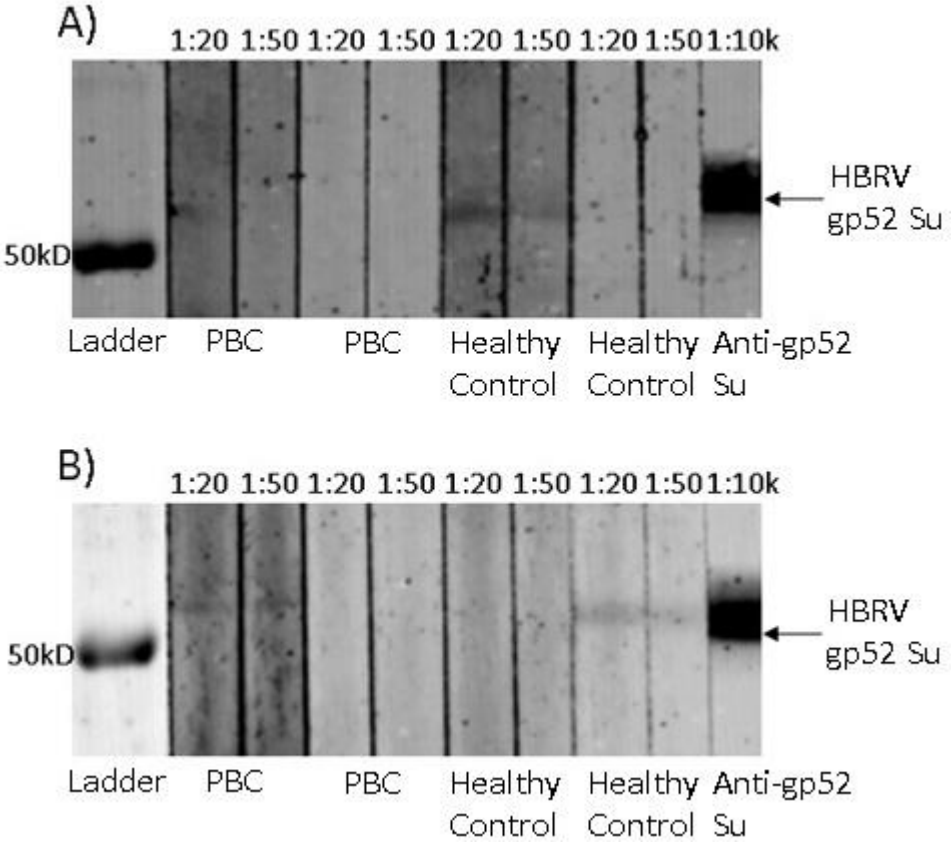
Purified HBRV gp52 Su expressed from mammalian cells was used in Western blots to test reactivity against PBC sera and healthy control sera from individuals whose samples had been previously utilized in as healthy controls in a breast cancer study. The anti-MMTV gp52 Su consistently exhibited a strong positive band at 52kD with no other banding. Immune purified Anti-Mitochondrial Antibody exhibited no reactivity, suggesting the purified gp52 Su was free of mitochondrial contaminants. Two samples showed reactivity at 52kD with no accessory bands: a PBC patient that was relatively highly reactive by ELISA, but was not a positive sample, and a healthy control with minimal reactivity by ELISA. A healthy control highly reactive by ELISA exhibited a distinct band at a slightly lower molecular weight than that expected for reactivity to gp52 Su, and as such was thought to represent reactivity not specific to gp52 Su.

A total of 42 patient samples were used to determine whether they exhibited anti-HBRV gp52 Su reactivity by Western blot. Patients were selected for testing by Western blot based on their reactivity to gp52 Su by ELISA in the HBRV gp52 Su ELISA version 3. Categorized based on diagnosis and reactivity by ELISA, the 11 most reactive of both the PBC patients and healthy control patients were tested, as were the 10 least reactivity of both groups. Of these patients, one highly reactive PBC patient (9.1%) and one low reactivity control patient (10%) produced banding by Western blot against HBRV gp52 Su. The PBC patient was not positive by the HBRV gp52 Su ELISA version 3, but was previously positive by the first and second versions of the ELISA utilizing mammalian antigen.

Figure 10:

HBRV Su Western Blots.

HBRV gp52 Su protein was resolved by SDS PAGE, and then transferred to PVDF membrane for Western blot. Individual strips were incubated with PBC and patient sera, developed and then assessed for bands at 52kD. 21 PBC patients, including 11 patients highly reactive to HBRV gp52 Su by ELISA and 10 patients with low reactivity to gp52 Su were examined. 21 health control patients were examined with 11 highly reactive patients by ELISA, and 10 patients with low reactivity. Patient sera was diluted to 1:20 and 1:50, while control anti-gp52 Su antibody was utilized at 1:10 000. Of the 42 tested patients, one PBC patient and one control patients exhibited banding to HBRV Su. One healthy control, pictured in A, exhibited banding at a slightly lower molecular weight than expected for anti-gp52 Su reactivity, which was thought to represent reactivity not specific to gp52 Su.



Chapter 4: Discussion

4.1 Introduction

We sought to establish whether patients with PBC produce humoral antibody responses against HBRV Su antigens. To answer this problem we developed a diagnostic ELISA assay to screen panels of PBC and healthy control patient serum; Western blot was then used as a second immunoassay method to validate the ELISA results. This diagnostic structure parallels other paradigms for diagnosis in other infectious human retroviruses such as HIV (4) and HTLV-1 (12). Another goal of the project was to develop a diagnostic ELISA for the detection of HBRV infection.

Our work began with the development of an expression construct for producing HBRV su truncations from *E. coli*. Of the four HBRV su proteins only Proteins 1, 3 and 4 were successfully expressed into the GST-HBRV Su fusion proteins. Antigen derived from Protein 3 exhibited the highest reactivity against anti-gp52 Su by ELISA. When utilized in an ELISA against PBC and healthy control patients, the assay initially showed a significant mean difference in the extent of reactivity towards HBRV gp52 Su between PBC and healthy control subjects. However, after scaling up the assay to a larger pool of serum samples, this difference disappeared. Subsequently, *E. coli* produced HBRV Su was abandoned in favour of HBRV gp52 Su expressed in human cells.

A stably transfected 293T cell line expressing HBRV Su was prepared by Dr. Guangzhi Zhang, a research associate in the Mason lab. The mammalian HBRV Su was purified utilizing a HIS-tag expressed as a fusion protein with the desired target protein. The HBRV gp52 Su ELISA version 1 utilized the mammalian expressed antigen and resulted in statistically significant higher levels of reactivity between PBC patient and healthy controls. Furthermore, the proportion of patients with a positive test was significantly different from PBC patients and controls (20% vs 4.5%). InBios, our collaborating partners, were also able to demonstrate a significant difference in the proportion of patients with a positive test versus controls with the HBRV gp52 Su ELISA version 2 (20% vs 3%). InBios also utilized a panel of PSC and non-biliary liver disease patients.

Attempts to replicate the InBios studies using a larger pool of PBC patients and healthy controls in the ELISA version 3 resulted in a lower overall detection rate and a lack of disease association. The handling of the HBRV Su used in the experiment, including introducing a number of freeze-thaw cycles, may have impacted the results by denaturing the antigen. As such, the InBios study likely reflects the strongest technical assay and thus most convincing result. However, using middle-aged women for comparison, as in the HBRV gp52 Su ELISA version 3, provides a more appropriate control group as approximately 90% of PBC patients are adult females. The HBRV gp52 Su ELISA studies have been recently extended for another project in the Mason Lab using ~120 samples showing that 8% of patients with breast cancer versus none of the age/sexed match controls demonstrated anti-gp52 Su reactivity. These studies used a similar protocol employed by InBios.

The Western blot studies provided little validation of my ELISA results. One highly reactive PBC patient and one non-reactive healthy control were demonstrated serological reactivity at 52kD, reflecting the HBRV gp52 Su. However, 40/42 (95.2%) of the samples tested by Western blot exhibited no banding specific to the mammalian expressed HBRV gp52 Su. ELISA detects conformational epitopes while Western blot detects linear epitopes. This may explain differences in the detection of positive samples between the assays. Notably, the Western blot used to confirm the data from the breast cancer study confirmed more positives (data not shown), suggesting that there may have been differences in the HBRV gp52 Su antigen used for this study.

4.2 HBRV Antigen Production

The expression constructs in *E. coli* offered a system in which large quantities of antigen could theoretically be produced rapidly for use in ELISA and Western blot assays. *E. coli* are amenable to high density culture, cultures are easy to scale-up, they exhibit rapid biomass accumulation (42) leading to high quantity recombinant protein expression and are rather simple to handle. The ability to rapidly expand *E. coli* cultures, express, then purify product over two days presented an attractive system.

Initial attempts to express protein from the HBRV su constructs at 37°C resulted in product mainly present in the insoluble fraction. Transitioning to a lower temperature (30°C) contributed to more successful expression. The presence of insoluble proteins when expressed at higher temperatures is likely due to presence of inclusion bodies result from hydrophobic interactions that are largely temperature dependent (42). Expression at reduced temperatures tends to result in reduced hydrophobic interactions, subsequently allowing correct folding patterns that improve stability. Lower degradation may be achieved at temperatures below 23°C; however, successful expression did not occur under these conditions.

We could not identify why we failed to produce HBRV Su Protein 2. Protein expression was attempted under a number of conditions, some of which lead to the successful production of the other proteins. As such, it seems unlikely the conditions of the expression were to blame. While it is possible some of the expressed sequence was toxic, leading to death of cells expressing the construct, this is unlikely as Protein 4 contained the full Protein 2 sequence. While it was not clear from Western blot that production of Protein 4 succeeded, as the expressed Protein 3 and 4 products appeared of similar size by Western blot, Protein 3 and 4 produced different reactivity to anti-gp52 Su by ELISA. This suggested that the two proteins were distinct antigens and thus expression of both likely succeeded. As the quality of the Construct 2 sequence was verified using sequencing, it is unlikely expression issues were the result of errors in the expression vector.

The benefit of being able to produce a large quantity of protein easily in the *E. coli* system was offset by the relatively small proportion of antigen that remained in the soluble fraction after sonication. Furthermore, the requirement to lyse the *E. coli* meant that the antigen was released along with all the other cellular contents. While GST-tag purification was utilized to collect the

product, SDS-page and Western blot results emphasized that even following purification, a great deal of non-gp52 Su contamination was not removed. Therefore, the use of this protein for Western blot work was limited without further purification steps, which themselves would result in a lower resultant concentration of purified antigen. The *E. coli* product also contained a great deal of degradation products, to the point where degradation product was of higher quantity than intact protein. This may present an issue in ELISA as relatively little of the intact protein may exist to present conformational epitopes to patient antibodies. Accordingly, the antigen retrieval could have been improved by utilizing a size selection column to reduce the degree to which degradation products were present in the final antigen preparation.

Use of the *E. coli* produced HBRV Su in ELISA meant that the patient sera had to be blocked using *E. coli* lysate. However, the project moved from *E. coli* HBRV Su to the mammalian antigen shortly after experiments with *E. coli* lysate blocking began, making it unnecessary to thoroughly optimize the *E. coli* lysate block. Blocking with quantities of 150-200ng of *E. coli* lysate from control BL21 cells had little discernible effect on sample readings. Blocking could have been improved by titrating a wide range of lysate concentrations to establish the optimal point at which anti- *E. coli* antibody signal could be blocked without interfering with anti-HBRV gp52 Su signal.

Ultimately, the *E. coli* expression system discontinued because the mammalian expression system was established by Dr. Guangzhi Zhang. The *E. coli* expression system exhibits shortcomings in protein glycosylation and protein refolding compared to mammalian expression systems (42). *E. coli* lack the cellular organelles required for glycosylation, even though non-enzymatic glycosylation of recombinant proteins may occur (30). As such, HBRV Su expressed in *E. coli* would likely not have the natural glycosylation found in mammalian cells. HBRV and MMTV exhibit a high degree of similarity (49). While MMTV Su only exhibits 3 glycosylation sites, relatively few compared to other retroviruses (7), these sites may to recognition by anti-HBRV gp52 Su antibodies.

Glycoproteins also aid protein folding (5). Through binding to the oligosaccharides of unfolded proteins, chaperone proteins in the endoplasmic reticulum prevent exporting of unfolded proteins until proper folding has been achieved. Therefore, expression of HBRV Su in mammalian cells would ensure proper folding of the antigen takes place. Further complications of producing

mammalian proteins in *E. coli* include non-specific disulfide-bond formation leading to aggregation of protein and codon bias reflecting the concern that specific tRNAs required for target protein production may be rare in *E. coli* (42).

Beyond the differences between mammalian and *E. coli* protein expression, the mammalian system was capable of producing HBRV Su secreted into serum free media, eliminating the issue of cellular contaminants of the target protein due to cell lysis. Furthermore, the use of serum free media for the protein collection improved the ability to purify the secreted HBRV gp52 Su. The presence of a stably transfected 293T cell line capable of producing HBRV gp52 Su also meant that the cells could be grown up in large quantities ensuring large-scale experiments could be completed using protein collected in the same batch under identical circumstances. As such, there was little reason to utilize the *E. coli* expression system when production of the mammalian HBRV gp52 Su succeeded in creating a much purer antigen.

In preparation for the HBRV gp52 Su ELISA version 1 study, the mammalian expressed antigen was purified and aliquoted for storage such that the antigen only underwent one freeze/thaw cycle before use. While effects of multiple freeze thaw cycles on the HBRV gp52 Su product was not tested, they were limited in anticipation that repeat freeze-thaw cycles may result in degradation or conformational changes.

In an aqueous environment, it is assumed that a protein's spontaneous folding places the hydrophobic amino-acids in such a fashion that they become encased by polar amino-acid side chains, which provide a hydration layer (36). However, the crystal lattice produced by freezing disrupts the hydration layer, allowing unfolding and subsequent hydrophobic interactions that denature the protein. Freezing acts as a physical denaturant (35). Two freeze-thaw cycles of HIV gp160 reduces CD4 binding by 50%, suggesting the freeze-thaw cycles interrupted the protein's native confirmation. As such, the same may hold true for HBRV gp52 Su.

In contrast to earlier ELISAs, Dr. Guangzhi Zhang provided the antigen used in the HBRV gp52 Su ELISA version 3 and validation Western blots. The purification scheme followed a similar design to the earlier strategy, however a simpler pump system was used in place of FPLC, which was utilized in purification of all mammalian produced HBRV gp52 Su used for versions 1 and 2 of the HBRV gp52 su ELISA. Furthermore, the protein was put through multiple freeze-thaw cycles during antigen preparation, which may have impacted the integrity of the protein

explaining some of the variation in findings between the assays. However, even with multiple freeze-thaw cycles, no degradation products were visible by SDS-PAGE with Coomassie blue staining, or by Western blot utilizing anti-gp52 Su.

4.3 ELISA Optimization

Optimizations utilized a checkerboard titration in a standard approach for ELISA development (9). Reagents such as the primary antibody or sera, secondary antibody, and antigen were diluted against each other to establish the optimal reactivity observed with each combination. In the *E. coli* ELISA, optimization efforts were complicated by the addition of *E. coli* blocking. In the HBRV gp52 Su ELISA version 1, initial attempts to include PDC-E2 blocking of AMA in sera samples increased the complexity of our efforts. In both ELISA designs, the lack of positive and negative patient controls led to the need to titrate various patient samples during optimization, greatly increasing the extent of the optimization process. Optimizations in all assays were limited by the small quantity of sera available for most patients.

The ELISA experiments with *E. coli* and mammalian produced antigens suffered from a lack of known positive and negative controls. While anti-MMTV antibodies were used as surrogate, we had no samples from patients with HBRV infection known to be producing anti-HBRV Su antibodies or patients conclusively infected with HBRV. Therefore, only experimental methods could select patients with possible HBRV infection to be used in the ELISA. Mandana Rahbari, a PhD student in the Mason lab, produced T cell epitope work that identified a number of patients with T cells responsive to peptide sequences derived from HBRV Gag and Env, suggestive of HBRV exposure. These patients were selected to provide samples for preliminary ELISA optimizations. Therefore, initial optimization experiments was based on the hypothesis that patients with T cell responses to HBRV Su epitopes may also have B cell responses, and therefore antibodies against to HBRV gp52 Su.

However, this assumption that PBC patients with T cell responses to HBRV will also have B cell responses to HBRV gp52 Su may be incorrect. Infectious agents may have immunomodulatory effects, which could extend to induction of T or B cell tolerance (9). Preliminary work by Mandana Rahbari suggested that antibody production by B cells in PBC patients may be suppressed (personal communications). The working hypothesis being explored is that

betaretrovirus Env engages TLR4, which subsequently leads to the production of IL-10, a suppressive cytokine (21, 38). Therefore, patients with positive T cell responses may not also produce B cell responses.

Patients were grouped according to whether they had PBC or were healthy controls. However, viral activity in PBC patients can only be established in 75% of PBC patients via RT-PCR in perihepatic lymph nodes (49). In contrast, only 24% of PBC patients have evidence of HBRV by RT-PCR in blood samples (29). In light of this, ELISA studies would optimally have focused on RT-PCR positive PBC patients as one group compared to healthy controls, while PBC patients negative by RT-PCR could have presented a third group for analysis. Diagnostic tests for HBV (48) utilized PCR testing for viral DNA to confirm viral infection in patients as a gold standard of diagnostic accuracy for ELISA to be tested against. While prior RT-PCR studies in PBC patients may have assisted in selecting HBRV infected patients, the detection frequency in blood is low.

For many patients serum, stocks were limited. As such the degree to which each reagent could be optimized was restricted. While these limitations did not significantly impact the optimization of the major reagents, it did limit the degree to which optimizations took place for *E. coli* lysate and PDC-E2 during the *E. coli* and mammalian ELISAs respectively. Having known positive and negative samples, as well as access to larger quantities of sera, would have allowed more extensive optimizations and possibly greater ability to distinguish between experimental groups based on the developed assay. As patients with highly reactive sera were more likely to be used in many optimization experiments, some of these patients' sera samples were exhausted. As such one highly reactive PBC patient sample from earlier ELISAs was not included in the HBRV gp52 Su ELISA version 3.

4.4 PDC-E2 Blocking

PBC patient AMA binds PDC-E2 (49). There was concern that potential mitochondrial contamination of the HBRV gp52 Su purification product could lead to false positive results or elevated background across PBC patient samples due to AMA reactivity. In previous Western blot studies, PDC-E2 was successfully used to block AMA reactivity in PBC patient sera (28)

and the same procedure was used in the ELISA in hopes it would reduce background reactivity in the assay.

Patient serum was incubated with PDC-E2 prior to use in the ELISA in the protocol of the HBRV gp52 Su ELISA version 1. However, Western blot experiments utilizing AMA against gp52 Su exhibited no banding, suggesting no contamination of the viral antigen with mitochondrial proteins. InBios also supported abandoning PDC-E2 blocking as it seemed inconsequential to the results of the assay. As such, PDC-E2 blocking was not used for ELISA work beyond the first version of the HBRV gp52 Su ELISA.

4.5 Samples Removed from Analysis

Five sera samples donated by laboratory technicians were included in the HBRV gp52 Su ELISA versions 1 and 2. However, these samples were removed from analysis over concerns that lab technicians who have worked with tumor producing mice have been found to produce humoral immunity to MMTV (14). Indeed, one lab worker had both T cell reactivity and anti-HBRV gp52 responses. As such, we decided lab technicians may be poor controls for the study, and these samples were removed from all ELISA analysis. Similarly, a presumed healthy control sample was subsequently found to be from an individual with a bleeding disorder without a specific diagnosis. This patient was also removed from ELISA analysis as they did not represent a healthy control.

4.6 ELISA Assays

As discussed, the HBRV gp52 Su ELISA utilizing *E. coli* expressed antigen was largely limited by the quality of the assay's antigen. Given the degree to which impurities contaminated the antigen, it is difficult to judge the potential of the assay, which was discontinued due to the production of a superior antigen from 293T cells.

Of note, all mammalian expressed HBRV gp52 Su ELISAs consistently demonstrated a significantly higher mean optical density for reactivity for PBC patients versus all control samples studies. While this may or may not be of relevance to the biology of HBRV infection, only the frequency of positive reactions are of importance to determine significance of ELISA

results, assessed by Fisher's exact test or Chi-square test. It is accepted practice to determine positivity by establishing a cutoff of mean+2x S.D. or mean+3x S.D. (9).

Optimization of the mammalian expressed HBRV gp52 Su ELISA was relatively limited compared to the optimizations for the *E. coli* ELISA. The initial experiments with mammalian antigen produced more marked statistical discrimination between groups than the *E. coli* ELISA, even with only limited optimization. In addition, a number of high and low reactive samples from the *E. coli* ELISA were used to optimize under the assumption that both antigens would elicit anti-gp52 Su antibody reactivity. Promising conditions for the ELISA were identified rather early in optimizations, and as such, work moved towards larger studies to confirm the initial studies as opposed to focusing on further optimizations. It is possible that returning to optimization steps for the mammalian gp52 Su could result in more effective reagent concentrations and more distinct statistical differences between the results of the experimental groups. This is especially true for optimization of secondary antibody concentrations, which was fairly limited.

The HBRV gp52 Su ELISA version 1 identified positive reactivity in 20% of PBC patients (n=71) versus 4.5% of the healthy controls (n=44) using the less stringent cutoff of mean+2x S.D. (P=0.0.026). Therefore, the results suggested that PBC patients produce humoral reactivity to HBRV Su. However, this relationship was only significant when utilizing the less stringent cutoff for positivity. Six control samples were removed from analysis; five for being lab technicians while the last had a bleeding disorder of unknown etiology. When the removed control were included in analysis, the results of the studies were non-significant by the mean+2x S.D. cutoff (P=1) and by T-test (P=0.092).

Positive reactivity in control patients may reflect false positives, but as previous studies have found evidence of HBRV infection in healthy patients (49), the control patient reactivity may be true positive humoral reactivity to HBRV Su. Therefore, the ability to discriminate between PBC and control patients may be confounded by infection in healthy individuals. Differences in antibody production may also be present between control groups based on environmental exposures.

As such, another test, such as RT-PCR, would be desirable to identify the degree to which infection occurs in PBC and control patients. With this knowledge, antibody detection could be

measured against other parameters suggestive of infection as opposed to diagnosis. If RT-PCR is unable to identify HBRV infection in 100% of the population, which is currently true (49), it may suggest that HBRV infection is present in only a subset of PBC patients, and thus a viral etiology may not apply to all cases of PBC. It is also possible that the virus is capable of immunomodulation or induction of tolerance that would prevent production of antibodies. This would further reduce the proportion of PBC patients that would produce anti-HBRV antibodies. The current model of segregating patients for analysis based on diagnosis likely underestimates the effectiveness of the ELISA in detecting anti-HBRV Su antibodies in patients.

Following the HBRV gp52 Su ELISA version 1, the materials and protocols for the ELISA were passed on to InBios for improvement of the assay. The same reagents were used but InBios employed their own proprietary wash solutions, and changed incubations of primary and secondary antibody to 1 hour at 37°C. Along with the healthy controls and other liver disease samples provided by the Mason lab, InBios also included their own in-house NHS healthy controls in the ELISA study.

When the HBRV gp52 Su ELISA version 2 results were analyzed pooling the samples, 20% of PBC patients (n=82) versus 3% of healthy controls (n=58) exceeded the mean+2x S.D. cut off (Fisher's exact test, P=0.0047). As such, this ELISA result supports the hypothesis that PBC patients produce humoral reactivity to HBRV antigens. A similar frequency (3%) of reactivity was observed in the other liver disease control patients. Of note in regards to the historical interest in the association of MMTV with breast cancer, the lone NASH patient positive by ELISA also developed breast cancer. A comparison of reactivity between the PBC, healthy control, PSC and non-biliary liver disease patients was significantly different (Chi-square test P=0.0011). However, the mean+3x S.D. cut off resulted in a non-significant result but this may be too stringent.

Five lab technician samples and one patient with a bleeding disorder were removed from the above analysis due to being inappropriate healthy controls. When these controls were included, the results comparing PBC patients and healthy controls by T test remained significant (P=0.031) while the Chi-squared test comparing all examined groups was non-significant (P=0.201).

A large-scale study was performed in the Mason lab using 102 sera from healthy women for controls as compared to 103 samples from PBC patients. A new pool of healthy controls

comprised of controls from breast cancer studies was used as the breast cancer study control more closely matched out PBC patients in age and sex than our previous control group. However, serological reactivity was only detected in 4% of PBC patients and 2% of healthy controls. The low number of positives identified by the HBRV gp52 Su ELISA version 3 relative to the other ELISAs may be due to differences in handling of the HBRV Su antigen. Previous ELISAs used gp52 Su that was aliquoted shortly after production so antigen underwent one freeze-thaw cycle only. The ELISA version 3 utilized antigen that underwent multiple freeze-thaw cycles, possibly resulting in denaturation and interruption of the protein's native conformation.

Comparisons of the results of each individual mammalian gp52 Su ELISA is difficult because each assay examined the problem under different conditions. Only sera concentration, secondary antibody concentration, and antigen quantity remained constant between all assays. Differing conditions between assays are summarized in Table 3.

Table 3: Incongruent HBRV gp52 Su ELISA Conditions

Condition	**Version 1**	**Version 2**	**Version 3**
Primary Antibody Incubation	Overnight at 4°C	One hour at 37°C	One hour at 37°C
Secondary Antibody Incubation	3 hours at room temperature	One hour at 37°C	One hour at 37°C
Control Group	Mason Healthy Controls	Mason Healthy Controls	Breast Cancer Study Healthy Controls
Wash Buffer	0.05% Tween20 in PBS	Proprietary InBIOS buffer	0.05% Tween20 in PBS
Standardization	1/40 000 Anti-gp52	1/4000 Anti-gp52	Standard Curve: 1:1000- 1:128000 Anti-gp52
Serum Freeze Thaw Cycles	Two	Two	Three
Antigen Freeze Thaw Cycles	One	One	Four

Furthermore, each assay utilized different patient groups. Even in the most comparable assays, versions 1 and 2 of the HBRV gp52 Su ELISA, the version 2 excluded 19/71 of the PBC serum samples included in version 1 and version 1 excluded 12/82 PBC samples that were included in

version 2. These omissions undoubtedly contributed to the differences in significance levels and positive samples detected between the two ELISAs.

Due to these differences, the ELISA results are best analyzed separately as three different ELISA approaches. Of the ELISAs, the ELISA version 2 was the most technically sound experiment as it balanced large groups with minimal antigen freeze thaw cycles. This ELISA was also run by experienced technicians utilizing proprietary materials and mechanized washing, which could not be fully replicated by our lab. Perhaps as a result, the ELISA version 2 produced the most convincing results of the ELISAs.

While version 3 of the HBRV gp52 Su ELISAs did not demonstrate significant differences, the ELISA version 1 and 2 found reactivity to HBRV gp52 Su is associated with PBC diagnosis. This suggested that some PBC patients produce antibodies to HBRV Su, although as some controls were also positive by the cutoffs used to establish positivity, this finding was not exclusive to PBC patients. The finding that control patients may also produce anti-HBRV Su antibodies is unsurprising, given the expectation that a portion of healthy individuals exhibit evidence of HBRV infection (49).

In summary, the HBRV gp52 Su version 2 results suggest that, at least under the conditions of the mean+2x S.D. cutoff of positivity, PBC patients exhibit higher reactivity to HBRV Su than healthy controls, PSC patients, and non-biliary liver disease patients. Furthermore, version 1 of the ELISA also found PBC patients exhibited higher reactivity to HBRV Su than did healthy controls. However, the significance of the above findings was not retained when analyzing data using a more stringent cutoff of positivity, suggesting the assay may not be useful as a diagnostic test – especially because only one in five PBC patients were found to make serological responses to HBRV gp52 Su, whereas using RT-PCR three of four patients have evidence of infection.

4.7 Western Blots

Western blots were used as a second immunoassay to validate the ELISA results. A portion of patients were selected for validation based on their reactivity to HBRV gp52 Su in the ELISA version 3 to ensure that reactivity displayed by high ELISA results was anti-gp52 Su reactivity, and not non-specific. Both high and low reacting PBC and healthy control patient samples were selected, specifically the most extreme samples of each group. The expectation was that if serum

activity exhibited in the ELISA was HBRV gp52 Su specific, patients with high reactivity to antigen in the ELISA, regardless of diagnosis, should exhibit anti-HBRV gp52 Su specific banding by Western blot.

Screening included 42 patient samples, including the 11 most reactive of both the PBC patients and healthy control patients, as well as the 10 least reactive samples of each group. Reactivity was judged by the standardized sample O.D. in the HBRV gp52 Su ELISA version 3. Of these samples, one highly reactive PBC sample displayed banding by Western while one healthy control patient that showed non-reactivity by ELISA also exhibited banding.

The results suggest that, at least under the tested conditions, the ELISA results cannot be conclusively validated via Western blot. One reason the ELISA and Western blot may be showing conflicting results for the same patient sample, with positive results from one assay type and negative from the other, is that the assays may be sampling different populations of antibodies. Some antibodies recognize conformational epitopes, or epitopes consisting of amino acids which are from non-sequential areas of a molecule (9). Therefore, the recognition of the antigen by the antibody is dependent on the antibody being present in its native three-dimensional structure. The ELISA presents antigen in its native conformation, meaning conformational isotopes will be present, while some linear epitopes may be hidden from antibodies. Western blot, in contrast, utilizes denatured protein. As such, conformational isotopes may not be present while linear epitopes will be.

However, ELISA screening followed by Western blot validation has been successfully used as a diagnostic paradigm in the case of other human retroviruses including HIV (4) and HTLV-1 (12). While some positive anti-HBRV Su reactivity was detected by Western, the assay was not extensively optimized. As such, further optimization, especially regarding quantity of HBRV Su loaded and incubation conditions, may result in detection of more HBRV Su reactive samples.

Optimally, an immunoassay utilized to validate the ELISA would be one in which the conformation of the antigen could be retained. Thus the two assays would both be sampling antibodies to the conformational epitopes of gp52 Su. A candidate assay would be a Western blot in non-denaturing conditions, which is a Western blot protocol without heating samples and with SDS excluded from sample and migration buffers. Beyond conformation, some antibodies may not recognize protein in a non-reduced (oxidized form). As such, non-reducing conditions should

be used in the loading and migration buffers. Thus, no reducing agents such as DTT and beta-mercaptoethanol should be added. Attempting validation of the ELISA results utilizing immunoassay methods that retain protein conformation may produce more relevant results.

While the HBRV Su Western blot did not adequately validate the ELISA results, it did exhibit a second means by which anti-HBRV Su antibody reactivity was successfully detected. While detection did occur, it was only in a small proportion of patients. This may be because most anti-HBRV gp52 Su antibodies are conformation specific, or may be due to the limited optimizations of Western conditions failing to achieve optimal experimental conditions.

4.8 Viral ELISA Diagnostics

Various viral diagnostic ELISAs utilize or avoid using envelope products as antigens. The third generation HIV IA-western blot utilizes gp160 (34), an Env region product (7). HTLV-1 gp46 is the HTLV-1 Su protein (7), which is used for Western blot and ELISA (47). Diagnostics for HBV also probe for anti-HBs reactive to HBV surface antigen that cannot be detected in active infection but are present following clearance of virus or after successful vaccination (39). HCV diagnostics forego testing for antibodies against HCV surface antigens, instead using 4 non-structural HCV antigens and HCV core antigen (1) to probe for their respective patient antibodies. HCV E1 and E2 are HCV envelope proteins (15). While the capability of anti-E1 and E2 antibodies to be elicited from HCV patient sera samples varies widely based on antigen preparation, anti-E1 and anti-E2 antibodies can be detected in up to 93% and 70% of chronic HCV patients, respectively.

HBRV Su was selected as a putative antigen due to the use of viral Env products in a number of viral diagnostics and tests for anti-viral humoral immunity. Furthermore, the first components of a pathogen recognized as foreign during infection of the mammalian body tend to be surface components (9). Anti-viral antibodies are mounted against Envelope and Capsid antigens of viruses because these proteins are exposed on the viral surface. Previous ELISA work featuring other viruses suggest *env* region expression products provide a promising target for sampling antibody populations. However, the HBRV gp52 Su ELISA only successfully produced an association between patient group and reactivity to HBRV Su in the first and second versions of

the assay, both while using the lowest cutoff of significance for analysis. As such, the assay must be improved to produce a more convincing result.

It is a trend that as diagnostic ELISAs for viruses are developed, more antigens are included. The odds of detection of positive cases is improved with the inclusion of greater numbers of antigens allowing sampling of greater antibody diversity (9). HIV immunoassays utilize gp160 Envelope protein along with the p24 Capsid protein, and Transmembrane protein antigen (34). HTLV-1 testing also utilizes Capsid and Su proteins (47). HCV immunoassays avoid use of Env products, but second generation HCV assays included 2 non-structural antigens and the HCV core antigen (1), while the third generation added a further non-structural HCV antigen. HBV immunoassays utilize surface antigen, core antigen, and the HBe antigen (39).

As such, there is a strong precedent for adding further antigens to improve ability of a test to detect antibodies to viral antigens. However, care must be taken in selection of antigens to avoid selecting antigens that produce cross-reaction (9). For example, 27/77 (35%) of PBC patients have been found to display humoral immunity to HIV-1 p24 Gag by western blot (27). This suggested that PBC patients were infected with a retrovirus, and produced antibodies that were cross-reactive to HIV-1 p24 Gag.

The addition of further antigens to the HBRV Su ELISA may produce an enhanced ability for the assay to detect antiviral antibodies in serum samples. However, care must be taken to avoid proteins that produce cross-reactivity.

4.9 HBRV Su ELISA as a Diagnostic Assay

Given the lack of significant statistical discrimination between PBC patients and healthy controls in the HBRV gp52 Su ELISA with a high stringency cutoff, it is likely unable to perform as a diagnostic assay. Indeed, in even the most promising assay, the HBRV gp52 Su ELISA version 2, only detected serological reactivity in 20% of PBC patients. Thus, the test had a sensitivity of 19.5% and a specificity of 96.6%. As such, the assay presents a poor screening tool, but provides very strong confirmation in those positive.

The ELISA version 2 results translate to a positive predictive value (PPV) of 88.8% and a negative predictive value (NPV) of 45.9%. Given that 75% of PBC patients exhibit HBRV RNA

by PCR (49), only detecting anti-HBRV Su in 19.5% of patients is very low. Anti-HBRV Su antibodies may present a poor target for diagnostics or the assay in its current iteration may be inadequate to detect anti-HBRV Su antibodies in most PBC patients despite antibody presence.

However, this analysis assumed that all PBC patients should be true positives for HBRV infection. RT-PCR is used to detect and quantify viral infection in serum in the case of hepatitis C (1) and PCR in the case of hepatitis B (48). However, 75% of PBC patients exhibit evidence of by RT- PCR in lymph nodes (49) and only 24% in blood samples (29); Therefore RT-PCR cannot be used as a gold standard to detect viral infection in PBC patients. Better diagnostic assays are required to detect HBRV infection.

While this ELISA had a low false positive rate, the poor NPV suggests that negative results are often untrue. Consequently the test had poor sensitivity. Considering the examined data represented by far the most promising ELISA results, the data suggests the HBRV gp52 Su ELISA cannot act as a diagnostic test. By comparison, the third generation HCV EIA had a specificity of 98.4% and a sensitivity of 98.1% (1). Even the 1st generation anti-HCV assay detected antibody in 68% of patients (2) compared to the detection of antibody in 19.5% of PBC patients by the HBRV Su ELISA. Extensive optimizations of the assay and inclusion of other viral antigens, such as gag proteins, in the assay may allow for increased ability to detect anti-viral humoral immunity. As such, the potential to utilize gp52 Su as part of a larger diagnostic paradigm should not be dismissed without further assay development

4.10 Do PBC patients Produce Anti-HBRV Su Antibodies: Relationship between Disease and Anti-HBRV Su Antibodies

The ELISAs performed under different conditions produced conflicting results of patient reactivity to HBRV Su. However, the HBRV gp52 Su ELISA version 2 was the most technically sound assay and produced some evidence of an association between patient diagnosis and reactivity to HBRV Su. Significant findings resulted from analysis of positive and negative patient reactivity to HBRV Su from healthy controls, PBC, PSC, and non-biliary liver disease patients. Along with version 2 of the ELISA, Version 1 also produced some evidence suggesting a diagnosis of PBC was associated with reactivity to HBRV Su. However, the findings of both studies were only significant while using the least stringent cut off of positivity.

While some ELISA findings supported an association between HRBV Su reactivity and PBC diagnosis, attempts to validate the ELISA findings with Western blot were limited. As such, further work is required to produce definitive conclusions on the association between PBC and humoral reactivity to HBRV antigens. However, banding was produced by one PBC patient and one healthy control, establishing that some PBC patients and controls produce antibodies to HBRV Su.

The HBRV gp52 Su ELISA detected significantly higher positivity in PBC patients compared to healthy controls in version 1 (19.7% vs 4.5%) and version 2 (19.5% vs 3.4%) of the assay. Therefore, at least under the conditions of these assays, a relationship between PBC and anti-HBRV Su antibodies was tentatively established. However, consistent replication of results is required to lend strength to this conclusion. Furthermore, the failure to confirm the results via Western blot highlights the need for a form of assay validation that retains the proteins conformation. By the version 2 ELISA, 16/82 PBC patients, 2/58 healthy controls, 0/28 PSC patients and 1/30 NASH and combined hepatitis patients exhibited positive responses to HBRV gp52 Su, suggesting there was an association between patient diagnosis and reactivity to HBRV Su. Notable in regards to previous work on a possible association between MMTV infection and breast cancer, the NASH patient with a positive reactivity to gp52 Su had breast cancer. The ELISA findings strengthened the evidence of an association between PBC and anti-gp52 Su reactivity, but do not resolve the lack of ELISA validation by a second immunoassay.

These data suggest two possible conclusions: 20% of PBC patients may be infected with HBRV, and thus produce antibody. However, this conclusion conflicts with existing evidence, such as that provided by established RT-PCR studies suggesting infection rates of 75% (49). The second conclusion is that only 20% of PBC patients produce anti-HBRV Su antibody despite a larger proportion of infected PBC patients. This conclusion supports the possible suppression of antibody response by the virus. Mandana Rahbari's preliminary work suggests antibody production by B cells is suppressed in PBC (personal communication), and as such, the second conclusion may well be correct. Another alternative is that the ELISA data are the result of artefact.

To address the latter concern, other tests are required to demonstrate serological reactivity to HBRV. By Western blot, research groups have produced conflicting results using the highly

related virus, MMTV, and PBC patient sera to probe for humoral reactivity. PBC patients have been exhibited to produce antibodies reactive to Mm5MT cytosolic extracts and purified extracellular MMTV (28). The authors produced bands at roughly 52kD, suggesting the MMTV gp52 Su is one of the reactive proteins. However, Selmi et al. found that PBC patient sera did not produce reactivity against MMTV by Western blot (46).

The HBRV Su Western blot exhibited that only a small minority of 1/21 PBC patients exhibit banding to HBRV gp52 Su. 1/21 healthy control patients exhibited banding to HBRV gp52 Su. Furthermore, the healthy control patient was not positive by ELISA. As such, the Western blot results did not validate the ELISA results. While it seems that some PBC patients and healthy controls do produce anti-HBRV Su humoral responses, the results suggest that humoral responses to denatured HBRV Su is indistinguishable between the groups by Western blot. However, it should be mentioned that this analysis assumed that all PBC patients should be true positives for HBRV infection.

The HBRV gp52 Su ELISA versions 1 and 2 established an association between PBC diagnosis and reactivity to HBRV Su. However, this result must be replicated and successfully validated by experimental means, such as non-denaturing immunoblotting, in order to conclusively answer whether patients with PBC produce humoral immunity to HBRV antigens.

4.11 Future Direction

Denaturing Western blot does not represent the optimal mode to validate the ELISA findings due to concerns antigen conformation could be essential to anti-HBRV Su antibody-antigen interactions. As such, a second immunoblotting method could be utilized in an attempt to validate the ELISA findings. The chosen immunoblotting would preferably test antigenicity under native conditions in order to resolve whether the ELISA results were HBRV Su specific. An alternative would be to test other HBRV antigens and further studies using Gag and non-structural proteins are underway in the Mason lab.

While the HBRV gp52 Su ELISA versions 1 and 2 suggested PBC diagnosis is associated with reactivity to HBRV gp52 Su, a conclusive result would require revision of the ELISA with additional antigens and successful validation of results. The use of age and sex matched controls and access to larger pools of serum samples must be achieved in order to increase statistical

power of the ELISA results. Rotating plates could also be utilized for incubations as they allow maximal contact between solid and liquid phase molecules (9). Rotating plates also allows performance of ELISAs without temperature considerations, although optimization would still need to occur utilizing the rotating plates for incubations.

Chapter 5: Conclusions

Our project goals were to discern whether PBC patients produced antibodies against HBRV antigens and to produce a diagnostic ELISA for detecting HBRV infection. We partially addressed the hypothesis that PBC patients develop humoral immunity to HBRV by ELISA but these data were not supported by our Western blot studies and we could not convincingly demonstrate a relation between PBC and serological responses to HBRV.

The most rigorous ELISA study, ELISA version 2, completed in collaboration with InBios and utilizing mammalian HBRV gp52 protein, serum panel, and ELISA protocols with their modifications, utilized a further cohort of NHS healthy controls and exhibited a significant association between PBC patients and reactivity to HBRV gp52 Su. The association held true when including PSC and non-biliary liver disease controls in analysis. Furthermore, the first version of the ELISA also produced a similar significant association when comparing PBC patient and healthy control reactivity to gp52 Su. These associations suggests at least some PBC patients produce anti-HBRV Su antibodies. However, Western blot studies failed to provide validation for the ELISA findings. The Western blots did succeed in establishing that some PBC patients and healthy controls produce anti-HBRV Su antibodies, but the proportion of reactive samples was small.

As such, the findings lend some support to the hypothesis in that a portion of PBC patients produce antibodies against HBRV Su; however, replication of the ELISA studies and validation by a second immunoassay are required to confirm our results. The current model of segregating patients for analysis based on diagnosis likely underestimates the effectiveness of the ELISA in detecting anti-HBRV antibodies in patients. Further work should replicate the established results and a secondary immunoassay that retains antigen conformation should be utilized to validate ELISA results.

Our assay cannot currently contribute to a diagnostic to detect HBRV infection as the sensitivity of the assay was insufficient even while utilizing the lowest accepted cut off of positivity (mean+2x S.D.). We were unable to produce an assay that could detect reactive patient samples that were then able to be validated through a second immunoassay method. Thus, we were unable to produce a diagnostic to detect HBRV infection at this juncture.

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