

Development of an Enzyme-Linked Immunosorbent Assay to Determine the Seroprevalence of  
Bovine Leukemia Virus Antibodies in Humans

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## Abstract

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Breast cancer is a leading cause of morbidity and mortality worldwide. About 5-10% of breast cancer cases are associated with hereditary factors (such as mutations of the BRCA-1 or BRCA-2 gene) [1], but the exact causes for most breast cancers are unknown. The remaining 90% of breast cancer cases may potentially be caused by external initiators such as radiation, chemical carcinogens, or infectious agents. Infectious agents cause about 23% of all malignancies in developing countries, and approximately 8% of malignancies in developed countries [2]. Currently, six viruses are causally associated with human cancers: Epstein-Barr virus (EBV), human T-lymphotropic virus type 1 (HTLV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), human papilloma virus (HPV), and human herpes virus 8 (HHV-8). No infectious agent has yet been causally associated with human breast cancer. One candidate virus is bovine leukemia virus (BLV), a retrovirus closely related to HTLV and the causative agent of enzootic bovine lymphosarcoma. In cattle, BLV is transmitted by transfer of infected lymphocytes via blood or milk. Humans are potentially exposed to BLV by consumption of cow's milk and meat.

One part of the process of proving that an infectious agent causes a particular disease is proving that the organism infects humans. Production of immunoglobulins specific to the organism is often evidence of infection. Utilizing an immunoblot assay with a chemiluminescent endpoint, Buehring et al. [5] demonstrated the presence of anti-BLVp24<sup>gag</sup> IgG, IgM, and IgA in human sera. Competition studies with pre-immune and immune goat sera further verified the human anti-BLV specificity. Since immunoblotting is a labor-intensive and inefficient method compared to ELISA, an ELISA assay was developed in this study to detect anti-BLVp24<sup>gag</sup> IgG and IgM in human serum and plasma samples. Receiver operator curve analysis was used to compare the ELISA to the immunoblot. The ELISA method developed here has poor ability (IgG AUC=0.51, IgM AUC= 0.56) to discriminate between people with and without antibodies to BLV p24<sup>gag</sup>, compared to immunoblot. ROC analysis is concordant with the unimodal IgG and IgM frequency distributions.

The cut-off value derived from ROC analysis for IgG is 77800 RLU, which gives a sensitivity of 76.47% and a specificity of 26.1%. The cut-off value for IgM is 26244 RLU, giving a sensitivity of 80.9% and a specificity of 28.05%. Initial competition studies with a

monoclonal anti-human secondary antibody indicates that the Assay may show specificity for the BLV p24 protein. However, an appropriate antigen control was not available to rule out non-specific reactions with the antigen matrix. Therefore, this ELISA needs further development to assure specificity of the assay for the recombinant BLV p24<sup>gag</sup> protein before solid conclusions can be made regarding the seroprevalence of BLV antibodies in humans.

However, this developmental stage ELISA was used to estimate the frequency of anti-BLVp24<sup>gag</sup> IgG and IgM in our study population, using the ROC derived cut-off points. In the 0-3 month age category, the frequency of IgG is 14.2%, declining to 0% by the age of 6 months. The frequency of IgM in the 0-3 month age group is 23.9%, increasing to 70% by 6 months of age. The presence of IgG in the 0-3 month age group is most likely maternal IgG that wanes by age 6 months, while IgM is indicative of the child's own immune response to a new antigen. The level of IgG peaks at 30-39 years of age, plateaus, and then begins to decrease at 60 years of age. IgM peaks in adolescence and then begins a slow decline after 30 years of age, but never falls below 60% prevalence. Continuing high IgM titers may be indicative of constant reexposure to BLV via dairy and meat consumption, or to episodes of viral reactivation [4]. The relatively high overall percentage of IgM seropositivity (73.3%) compared to IgG seropositivity (49.3%) may be due to the low median age of the study population.

Using the preliminary ELISA method being developed here, this study may indicate that both vertical and sexual transmission of BLV potentially occur. However, presence of antibodies to the BLV p24<sup>gag</sup> protein do not necessarily indicate infection, but may result from exposure to the antigen from consumption of dairy products. In addition, confirmation of the specificity of this ELISA is necessary to make firm conclusions. Further prospective studies are warranted in order to determine precisely at what point(s) in the perinatal period vertical transmission could occur, and to elucidate whether sexual transmission is truly occurring. Prospective studies may also permit a study sample more representative of the target population to be developed.

In conclusion, widespread consumption of BLV-contaminated dairy and beef may potentially be responsible for a significant proportion of breast cancer cases worldwide. If the pathogenicity of BLV for humans is established, the implications are far-reaching and may indicate the need for primary preventative measures to avert continuing infection of humans.

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# Chapter 1: Background

## Overview

Breast cancer is a leading cause of morbidity and mortality worldwide. About 5-10% of breast cancer cases are associated with hereditary factors (such as mutations of the BRCA-1 or BRCA-2 gene) [1], but the exact causes for most breast cancers are unknown. The remaining 90% of breast cancer cases may potentially be caused by external initiators such as radiation, chemical carcinogens, or infectious agents. Infectious agents cause about 23% of all malignancies in developing countries, and approximately 8% of malignancies in developed countries [2]. Currently, six viruses are causally associated with human cancers: Epstein-Barr virus (EBV), human T-lymphotropic virus type 1 (HTLV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), human papilloma virus (HPV), and human herpes virus 8 (HHV-8). No infectious agent has yet been causally associated with human breast cancer. One candidate virus is bovine leukemia virus (BLV), a retrovirus closely related to HTLV and the causative agent of enzootic bovine lymphosarcoma. In cattle, BLV is transmitted by transfer of infected lymphocytes via blood or milk. Humans are potentially exposed to BLV by consumption of cow's milk and meat.

Establishing causality for oncogenic viruses requires multiple lines of evidence from biological and epidemiological studies. The presence of the virus in the tissue must first be demonstrated in the tissue in question, then established as more frequent in malignant versus normal tissue, and preceding the diagnosis of the cancer. This must be corroborated independently by other researchers. The virus should cause the cancer in an animal model or malignantly transform human cells in vitro. There should be a plausible reservoir and mode of transmission. Ongoing laboratory studies are providing evidence supporting the presence of BLV capsid protein and proviral DNA in human breast tissue [3]. The long latency period of malignant diseases makes prospective epidemiological studies problematic, however, one case-control study has suggested that BLV proviral DNA in human breast tissues is associated with breast cancer [4].

Although BLV would presumably be acquired by humans from consuming foodstuffs from infected cattle, no exact route of transmission has been established. If BLV causes breast cancer, then at what age are humans infected? How can the age of first exposure be detected? Knowledge of the basic properties of human immunoglobulins are the key. IgM antibodies are usually indicative of recent exposure, whereas IgG antibodies are indicative of past exposure either recent or many years before. Up until about 6 months of age, IgG antibodies may also be maternal antibodies that have passed through the placenta to the fetus, or have been transferred during via breastfeeding. In addition, as human infants mature and pass into childhood and young adulthood, life experiences expose one to different routes of transmission. Therefore, the age of first exposure can help determine whether an agent is transmitted vertically from mother to infant, or horizontally from person to person, either non-sexually or sexually. A seroprevalence study to determine the age of first exposure to BLV, using human anti-BLV IgG and IgM as the seromarkers, might be useful to elucidate transmission patterns at the population level. In 2003, the first published report of BLV antibodies in humans utilized a sensitive immunoblot technique [5]. Since immunoblotting is a labor-intensive and inefficient method compared to enzyme-linked immunosorbent assays (ELISAs), this study aims to develop an ELISA to analyze bovine leukemia virus antibody patterns in human sera. The seroprevalence

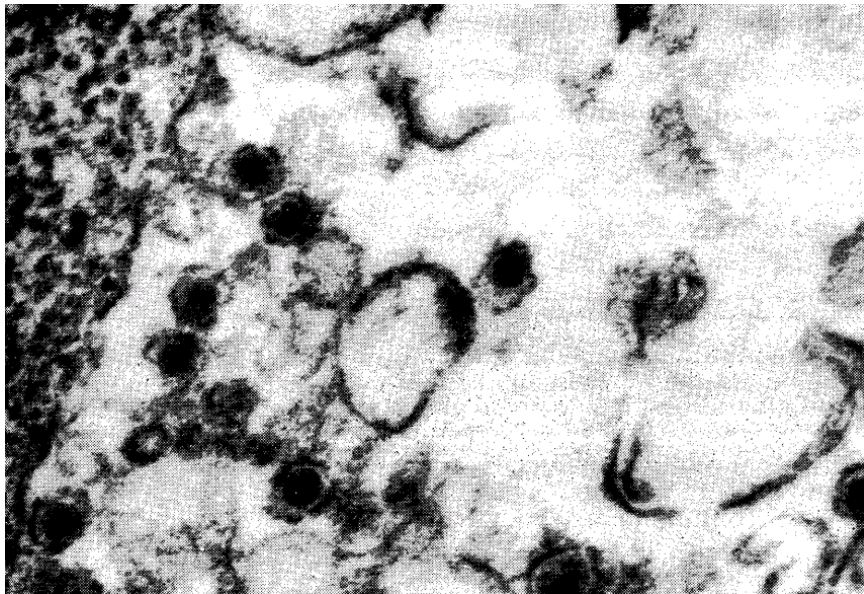
data obtained will then be statistically modeled to study age-dependency, which can be useful for determining routes of transmission. Existence of a transmission pattern not only provides further evidence that BLV infects humans, but would also provide the basis for prevention and control methods that could potentially reduce the burden of breast cancer disease globally.

### **History of Bovine Leukemia Virus**

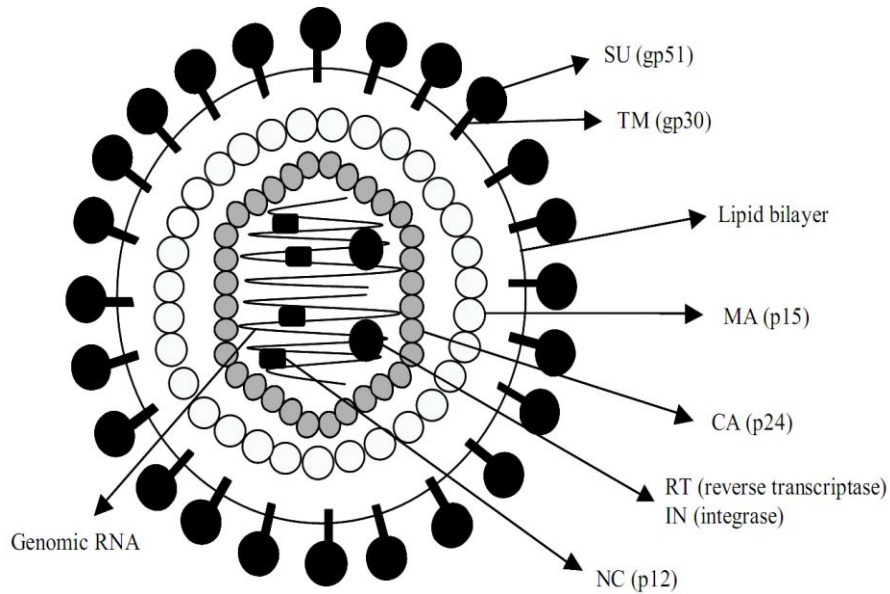
Enzootic bovine lymphosarcoma was first reported in Eastern Europe at the end of the nineteenth century, and in 1917 was shown to be transmissible by an infectious agent [6]. In 1969, Miller et al. demonstrated viral particles in lymphocytes from lymphosarcomatous cows by electron microscopy (Figure 1) [7]. Other investigators successfully replicated Miller's work, confirming the association between the virus and bovine lymphosarcoma. A causal association between the virus—now named bovine leukemia virus (BLV)—and enzootic bovine lymphosarcoma was demonstrated without difficulty by fulfilling Koch's postulates [8, 9].

### **Bovine Leukemia Virus Biology**

Bovine leukemia virus is an enveloped ssRNA virus (Figure 2) in the Family *Retroviridae*, Subfamily *Orthoretrovirinae*, Genus *Deltaretrovirus* [10]. BLV shares this genus with oncogenic human and simian T-cell lymphotropic viruses.



**Figure 1.** The first electron micrograph demonstrating bovine leukemia virus particles. [7].



**Figure 2.** Diagrammatic representation of the BLV viral particle [11].

BLV primarily infects B lymphocytes expressing surface immunoglobulin M (IgM) [11, 12]. Cells of the monocyte/macrophage lineage may also be infected [12], as well as T-lymphocytic cells [13], endothelial cells [14], and mammary epithelial cells [15]. After entering the target cell, the BLV RNA genome is converted to DNA in the host cell cytoplasm and may be integrated randomly into the host genome to form a provirus or remain in the cytoplasm as an episome. A persistent latent infection develops as a result of a bovine plasma factor that blocks viral expression at the transcriptional level [16]. Infected cells often express the CD5 molecule, and it is predominantly this IgM+/CD5+ population that expands resulting in a lymphocytosis [11]. The precise mechanism of transformation has not yet been elucidated, but evidence suggests that the virus inhibits host cell DNA repair mechanisms [17-19].

### **Transmission and epidemiology**

Natural transmission of BLV occurs only in cattle, zebus, buffalos, and capybaras [6]. Sheep and goats can be infected experimentally, but sheep develop cancer the most rapidly and are employed routinely for BLV research (reviewed in [20]). Evidence suggested that chimpanzees are also susceptible, but further studies were not pursued [21, 22].

The primary mode of BLV transmission in bovines occurs by transfer of infected lymphocytes in blood. Parenteral transmission often occurs as a result of certain agricultural practices such as unhygienic methods of dehorning, vaccinating, and tattooing which involve blood contamination of the instruments. These practices contribute significantly to the spread of BLV and increase the global burden of the disease. In addition, approximately 10% of calves contract the virus in utero [23]. Colostrum from BLV-positive cows may contain viral particles

and lead to further new infections, but maternal antibodies often protect against infection [24-27]. Infection results in life-long persistence, and there is no vaccine to prevent BLV transmission.

BLV infection is endemic in many cattle-raising countries around the globe. In the United States, for example, the 2007 National Animal Health Survey (NAHMS) estimated that 100% of large U.S. dairy operations were positive for BLV antibodies, as measured by ELISA of bulk milk [28]. Beef cattle are less likely to be infected with BLV, as shown in a study of Kansas beef bulls that calculated the estimated prevalence of 8.5% [29]. Most cattle produce a strong humoral response, and only 1-5% of cattle infected with the virus eventually develop lymphosarcoma [6]. Development of clinical signs and symptoms is dependent on the location of tumor formation, and will also vary according to tumor site. The most common site appears to be the digestive tract, resulting in anorexia and weight loss [30, 31]. Only the infected cattle that develop visually detectable clinical signs and symptoms of lymphosarcoma are culled from the herd and condemned at slaughter. The majority of infected cattle is apparently healthy and remains in the food system. Therefore, the virus is present in bovine milk [32, 33] and presumably meat products consumed by humans, raising the possibility that the BLV could pose a public health threat to humans.

### **Human Exposure to Bovine Leukemia Virus**

Extrapolating from studies on other food borne pathogens, inactivation of BLV in cooked meat is assumed to occur, but has never been tested directly. Sheep studies of the infectivity of cow's milk were accepted as evidence that pasteurization inactivates BLV [34-36]. Although most humans in the USA drink pasteurized milk, consumption of raw milk products is prevalent in some population groups. For example, an estimated 35-60% of farm families and farm employees consume raw milk [37]. A survey in California estimated that 3.2% of the study population consumed raw milk in the previous year, consistent with nationwide estimates [38, 39]. While the risk of contracting an infectious disease may increase with routine exposure, in theory only one exposure is needed for infection to occur. Therefore, persons who do not routinely and intentionally consume raw dairy could be exposed at some point during their lives.

### **Implications**

Biomedical and epidemiological studies on human breast tissues have provided evidence that BLV may not only infect humans but may also cause disease. In situ PCR and immunohistochemical techniques were used to investigate the presence of BLV proviral DNA and viral proteins, respectively, in human breast tissues. Proviral DNA sequences were detected, and a case-control study suggested that the presence of these sequences was significantly associated with breast cancer [3, 4]. Successfully controlling emerging infectious diseases requires knowledge of disease transmission mechanisms, whether at the molecular or the population level. Seroepidemiological studies that examine the age-dependency of specific antibody profiles are useful in determining transmission modes at the population level. The research presented here is an initial step in determining the age at which transmission of BLV to humans occurs and assessing human-human vertical transmission.

## Chapter 2: Assay Development

### Introduction

After the discovery of BLV in 1969, many seroepidemiological studies sought evidence of BLV infection in humans. These early studies (focused on potentially susceptible/exposed groups such as farmers, veterinarians, and rural oncology patients) were unsuccessful in finding serological evidence that humans were infected with BLV (reviewed in [40-42]). However, serological methods available at the time (complement fixation, agarose gel immunodiffusion, whole cell immunofluorescence) lacked the sensitivity and/or specificity that technologies today can achieve.

The first reliable study demonstrating antibodies to BLV in human sera was published in 2003. Utilizing an immunoblot assay with a chemiluminescent endpoint, Buehring et al. [5] demonstrated the presence of anti-BLVp24 IgG, IgM, and IgA in human sera. The study group comprised 257 volunteers recruited from the University of California, Berkeley, campus and the surrounding community. The use of recombinant p24 antigen improved the specificity of the reactions as compared to assays using viral lysate. Competition studies with pre-immune and immune goat sera further verified the human anti-BLV specificity.

However, since immunoblotting is a labor-intensive and inefficient method compared to ELISA, an ELISA assay was developed in this study to detect anti-BLV IgG and IgM in human serum and plasma samples. The ELISA method was compared to the immunoblot to determine assay performance parameters including clinical sensitivity and specificity.

### Materials and Methods

#### *Human samples*

Donor samples from three different study populations were used to develop and validate the ELISA. First and primarily, serum samples for development of this assay were obtained from the previous BLV immunoblot study [5]. Briefly, samples were obtained by venipuncture from adult volunteers solicited from the Berkeley community. Blood was drawn into serum separator tubes with clot activator and without anticoagulant. After centrifugation (500 x g), serum was stored in cryovials at 4°C until use. Second, samples positive and negative for BLV anti-p24 by dotblot (data unpublished) were used. Third, at the time of this study, blood tubes containing the anti-coagulant ethylene diamine tetraacetic acid (EDTA) were used to collect plasma samples by venipuncture from pediatric (newborn -18 years) donors at Kaiser Permanente, Oakland, CA. The protocol for acquisition for all specimens was approved by the UCB Committee for the Protection of Human subjects (protocol number #2010-10-2396).

### ***Specimen physical integrity***

At the time of this study, 10% of the archived serum samples from the original immunoblot study (n=25) were tested for cytomegalovirus (CMV) IgG antibodies (Wampole Laboratories, Inc, Princeton, NJ). CMV IgG testing was performed by a local clinical virology laboratory that runs the assay on a daily basis. The same samples were also tested in-house for IgG antibodies to the tetanus toxoid vaccine (Tetanus Toxoid IgG, ELISA KIT catalog #930-100-TTH, Alpha Diagnostic International, San Antonio, TX).

Representative serum specimens used for the assay development were diluted 1/2200 in wash buffer and used as the antigen for the immunoblot procedure to test for the presence of intact human immunoglobulins. The human samples were immunoblotted as previously described [5], and reacted with goat anti-human IgG, IgM, and IgA diluted 1/1000 in wash buffer.

### ***Enzyme linked immunosorbent assay (ELISA)***

Except as noted, all steps of the ELISA were performed at room temperature (22°C), with a volume of 200µl. Opaque, white, high-binding 96-well microtiter plates (Reacti-Bind™, ThermoScientific, Rockford, IL) were coated for two hours with 2.5 µg/ml of a recombinant BLV-p24 antigen in 0.1M carbonate-bicarbonate coating buffer (pH10.6). The recombinant p24 protein consists of 224 amino acids, with a pKa of 10.2. It was expressed in *E. coli* and purified on a nickel sepharose column followed by Biogel100 (p24 provided by Darrel Peterson, Department of Biochemistry & Molecular Biology, Virginia Commonwealth University). Plate wells were washed three times with wash buffer (Dulbecco's phosphate-buffered saline (DPBS) with 0.04% Tween-20) after all steps in the assay, except the blocking and detection stages. Non-specific binding sites were blocked with 250µl of an animal-product free blocking buffer (StabilGuard®, SurModics BioFxlaboratories, Owings Mill, MD) for one hour. Primary antibody (human samples) diluted 1/100 in wash buffer was reacted for two hours. Biotinylated goat anti-human IgG, IgM, and IgA secondary antibody (Vector Laboratories, Burlingame, CA) diluted to 5.0 x 10<sup>-5</sup>mg/ml in wash buffer was reacted for 90 minutes. Conjugate (streptavidin-horse radish peroxidase, Vector Laboratories, Burlingame, CA) diluted 1/2000 in wash buffer was incubated for 30 minutes. After a final wash, ultra-sensitive chemiluminescent detection reagent (SurModics BioFxlaboratories, Owings Mill, MD) was reacted for 5 minutes. Light emission was measured in relative light units (RLUs) in the SpectraMax M2 (Molecular Devices, Sunnyvale, CA). Human samples and controls were assayed in duplicate on the same plate in non-contiguous wells on the same day. Pre-immune and immune sera from the same goat (pre-immune catalog number 765198, immune catalog number 765026, National Cancer Institute, Biological Carcinogenesis Branch, distributed by Quality Biotech, Camden, NJ), were used as positive and negative controls with each run. Mouse monoclonal anti-BLV p24 produced in hybridoma medium by a hybridoma developed by Kramme et al., [15] diluted 1/1,000 in wash buffer, served as a second positive control. Rabbit anti-goat and horse anti-mouse secondary antibodies (IgG) (Vector Laboratories, Burlingame, CA) were diluted to 5.0 x 10<sup>-5</sup> mg/ml in wash buffer. Control wells, utilizing all assay components except for primary antibody, for which wash buffer was substituted, were included for each species. The RLU value of the appropriate blank was subtracted from the sample RLUs. This residual value was used to calculate the signal to noise ratio (SNR): SNR = RLU for positive sample/RLU for negative sample.

## ***Optimization***

Assay parameters were optimized by the checkerboard titration (CBT) approach. Preliminary testing was performed with 0.05M carbonate-bicarbonate coating buffer at pH9.6 and goat anti-human IgG, in 50µl, 100µl, and 200µl volumes. Recombinant p24 antigen was tested in a concentration range of 1-7.5µg/mL. The range of primary antibody dilutions tested was 1/20-1/1000, while secondary antibody was tested at 1/50-1/200,000 (0.01mg/ml-  $2.5 \times 10^{-6}$  mg/ml). Conjugate was tested from  $2.0 \times 10^{-3}$  mg/ml-  $4 \times 10^{-5}$  mg/ml. Various paired serum samples positive or negative for anti-BLV by immunoblot, or paired plasma samples positive or negative by dot blot, were used to determine the signal-to-noise ratio of the assay. The combination of assay parameters that yielded the highest SNR was selected for further optimization. The effects of pH and molarity on the SNR were then tested using coating buffer at pH 9.6 and pH 10.6, and molarities ranging from 0.05-0.1M. Finally, anti-human IgM and IgA were optimized with 2.5µg/ml p24 in 0.1M carbonate-bicarbonate coating buffer, pH10.6. The combination of assay parameters that yielded the highest SNR was selected for further validation.

## ***Validation***

### *Specificity*

To compare the performance of serum versus plasma samples, paired serum and EDTA plasma samples were obtained from pediatric donors (n=10) who had blood drawn by venipuncture for both a complete blood count and a chemistry test. The samples were tested for anti-BLV IgG using optimized assay parameters.

In addition, the effects of hemolysis on the ELISA IgG and IgM signals of the pediatric study group were investigated. First, a two sample t-test with equal variances was performed on the IgG and IgM signals of the hemolyzed and non-hemolyzed samples from the 0-1 month old pediatric group. Second, a comparison of hemolyzed and non-hemolyzed plasma from the same donor and same collection was performed. Whole EDTA blood was obtained from three separate donors. A 1ml aliquot from each donor was frozen in order to lyse the red blood cells and release the hemoglobin, hemolyzing the plasma. The remaining whole blood was centrifuged and separated without freezing in order to obtain non-hemolyzed plasma. The IgG and IgM signals of the hemolyzed and non-hemolyzed plasma was compared using a single-tailed paired student's t-test.

To determine the effects of non-immunoglobulin serum proteins on assay performance, paired serum samples and controls were treated with Melon™ Gel (ThermoScientific, Rockford, IL) according to manufacturer's guidelines. Melon™ Gel binds non-immunoglobulin serum proteins such as albumin from a variety of species including human, mouse and goat, using a chemical-based fractionation process at physiological pH. Most IgM and IgA is also bound, thus purifying IgG. CBT assay parameters for Melon™ Gel testing were as follows: 1.0µg/ml, 5.0 µg/ml, and 7.5 µg/ml p24; 1/100 human serum; 1/10,000 primary goat serum; anti-human IgG at  $2 \times 10^{-4}$  mg/ml and  $5.0 \times 10^{-5}$  mg/ml; and  $5 \times 10^{-4}$  mg/ml conjugate. The same paired sera were tested treated and untreated.

Competition studies were used to assess biological specificity. Human samples tested included serum both positive and negative on immunoblot, human serum treated and untreated with Melon™ Gel, and newly collected EDTA plasma samples from the pediatric donor study group. Human samples were diluted 1/100 in wash buffer. Pre-immune and immune goat sera (or the Melon™ Gel treated goat sera) were diluted 1/100 in wash buffer and then serially diluted in the human primary antibody preparation. Pre-immune and immune sera from the same sheep were used for some of the studies (pre-immune catalog number 765668, immune catalog number 795047, National Cancer Institute, Biological Carcinogenesis Branch, distributed by Quality Biotech, Camden, NJ). The secondary antibody was either polyclonal goat anti-human IgG or monoclonal mouse anti-human IgG<sub>1</sub> (Invitrogen, Grand Island, NY). ELISA was performed as described above. Neither secondary antibody was treated with Melon™ Gel.

To rule out the presence of anti-BLVp24 in the goat anti-human secondary IgG and IgM antibody preparations, the secondary antibodies were used as the primary antibody (diluted 1/1000 and 1/10,000 in wash buffer) in a routine ELISA assay. Rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA) was used as the secondary antibody.

Cross-reactions from antibodies to other viral agents causing chronic human disease were also evaluated. Sera from donors testing positive and negative for hepatitis C virus (HCV), human immunodeficiency virus (HIV), human T-lymphotropic virus-1 (HTLV-1), hepatitis B surface antigen (HbsAg), and cytomegalovirus (CMV) were run according the routine ELISA protocol. These sera were received from a large clinical laboratory performing testing on a daily basis.

### *Precision*

Assessment of assay precision was based on National Institutes of Health (NIH) reproducibility testing protocols and guidelines [43]. The results of precision and reproducibility testing reflect daily variation stemming from differences in plates, reagents, environmental conditions, and operator performance. Serum samples with low (L), medium (M), and high (H) signals, measured in relative light units (RLUs) in preliminary testing were tested on four different days in an interleaved plate format, rotating each sample through each position on the microtiter plate. Summary statistics (mean, standard deviation, percent coefficient of variation (CV %)) were calculated to quantify intra- and inter-plate variability. The acceptable range for intra-plate variability is <10% CV for each signal (L, M, H). The inter-plate acceptance criterion is ≤20% CV for each signal (L, M, H) and <2% outliers. The signal window (SW), a measure of the separation between the minimum and maximum signals, was also computed for each day (acceptance criteria = SW ≥2). A spatial uniformity assessment, consisting of a scatter plot of each signal by well number, was used to evaluate systematic trends such as drift and edge effects. Drift or edge effects, if present, should be <20%.

### *Determination of the cut-off value*

All remaining sera (n=253) from the BLV-EPI study were assayed for IgG and IgM anti-BLV antibodies using the optimized ELISA method. Receiver-operator curve (ROC) analysis using the samples' immunoblot results as the "gold standard" was the discriminatory test performed to determine the optimal cut-off values for IgG and IgM.

### Statistical Analysis

Statistical analyses were performed with the Stata software package (Stata, version 10) and with Excel (Microsoft Office 2007 and 2010). All statistical analyses were performed at  $\alpha=0.05$ .

## Results

### Specimen Physical Integrity

Two of the 25 samples submitted for CMV IgG testing were positive (8%). Twenty-four of the twenty-five samples (96%) were positive for IgG antibodies to tetanus toxoid. On immunoblot, bands were present for IgG, IgM, and IgA (data not shown).

### Optimization

For all secondary antibody and p24 concentrations tested with the coating buffer at 0.05M and pH 9.6, the anti-human IgG SNR ranged from 0.55-1.68, while the anti-goat IgG SNR ranged from 15-44. The best combination from this initial testing was selected for further coating buffer optimization: 2.5 $\mu$ g/ml p24 and anti-human IgG at 5 X 10<sup>-5</sup> mg/ml. Significant differences in SNR were seen with coating buffer at various pH and molarity (Table 1). The coating buffer formulation selected was 0.1M carbonate-bicarbonate, pH10.6, since the SNR was significantly higher than the other combinations. For a secondary antibody test range of 2.5 X 10<sup>-3</sup> – 5 X 10<sup>-5</sup> mg/ml, the anti-human IgM SNR was 0.47-8.22 and the anti-goat IgG SNR was 20.19-30.55. For a secondary antibody test range of 2.5 X 10<sup>-3</sup> – 5 X 10<sup>-5</sup> mg/ml, the anti-human IgA SNR was 0.48-2.36 and the anti-goat IgG SNR was 14.27-26.32. Based on this testing, a secondary concentration of 5 X 10<sup>-5</sup> mg/ml was selected for all three isotypes.

Coating Buffer	0.05M carbonate-bicarbonate		0.1M carbonate-bicarbonate		0.2M carbonate-bicarbonate	
	pH9.6	pH 10.6	pH9.6	pH 10.6	pH9.6	pH 10.6
human SNR Mean (95% CI)	1.48 (1.50-1.46)	1.68 (1.63-1.73)	9.51 (10.03-9.11)	17.96 (16.10-20.23)	13.25 (10.74-15.75)	6.02 (6.08-5.97)
goat SNR Mean (95% CI)	16.27 (16.64-15.93)	18.23 (22.93-15.20)	22.84 (24.61-21.38)	15.67 (15.86-15.50)	17.51 (23.72-13.88)	11.36 (11.60-11.13)

**Table 1.** Effect of coating buffer pH and molarity on signal-to-noise ratio (SNR).

## **Validation**

### *Specificity*

There was no significant difference in RLUs between serum and EDTA plasma (t-tests 0.14-0.84  $p = 0.05$ ). In pediatric donors aged less than one month, the mean IgG signal for hemolyzed samples ( $n=7$ ) was 99639.43 (95% CI: 17703.73-181575.10), while for non-hemolyzed samples ( $n=51$ ) in the same age group the mean IgG signal was 50265.02 (95% CI: 40970.42-59559.62), a statistically significant difference ( $p = 0.006$ ). There was no statistical difference for IgM for the same age group ( $p = 0.710$ ). In paired testing of hemolyzed and non-hemolyzed plasma from the same donor, two of three hemolyzed IgG signals were significantly lower than the non-hemolyzed signal (single-tailed paired student's t-test,  $p= 0.074, 0.030, 0.009$ ). All three hemolyzed IgM signals were significantly lower than the non-hemolyzed signal (single-tailed paired student's t-test,  $p= 0.005, 0.039, 0.029$ ).

The Melon™ Gel anti-human IgG SNR was 1.30-2.31, while untreated anti-human IgG SNR was 1.83-2.5. There was no significant difference between untreated and treated human sample SNR (two-tailed paired t-test: 0.052-0.128 ). The Melon™ Gel treated immune goat serum SNR was 60.79-140.15, while the untreated goat sera SNR was 16.74-27.74. The treated goat serum SNR was significantly higher than the untreated goat serum SNR (two-tailed paired t-test: 0.004-0.001 ).

There was no significant difference between the mean signals of the goat anti-human IgG (two-tailed student's t-test with unequal variance  $p=0.402, 0.146$ ) or IgM secondary antibodies (two-tailed student's t-test with unequal variance  $p=0.069, 0.617$ ) when used as a primary antibody, and the blank.

Virus cross-reactivity ELISA IgG results are displayed in Table 2. The mean RLU indicating antibodies to BLV in serum with antibodies to HIV and CMV, respectively, was significantly higher than the mean RLU of their respective negative sera. There was no significant difference for HTLV, HbsAg, or HCV. With an ELISA IgG cut-off point of 77800 RLUs, the mean signal of samples positive for all of the human viruses for which antibodies were tested, would be interpreted as positive for anti-BLVp24 IgG.

Competition studies indicated that immune goat serum did not successfully compete with the IgG reaction of the human serum (Figure 3a); Melon™ Gel treated goat serum did not compete with Melon™ Gel treated human serum (Figure 3b); nor did immune sheep serum compete with human serum (Figure 3c), when using a polyclonal goat anti-human IgG secondary antibody. Initial testing using the mouse monoclonal as the secondary antibody (Figures 3d, 3e) however, shows competition by the immune goat and sheep sera was higher than that by the naïve goat and sheep sera at high competing serum concentrations. A convergence of the two curves occurs as the concentrations of animal sera diminished, what would be expected for successful competition. A similar but less pronounced effect occurred with serum # 27, which was negative by IB. This suggests it may be a weak positive sample rather than completely negative, or that there may be some degree of non-specific reactions. In the competition study

using sheep sera (Figure 3e), the curve for the immune serum series demonstrates a minimal increase in signal with increasing concentration of human serum. Further studies are needed to clarify the dynamics of this competition system.

Virus Antibody	mean IgG RLU	anti-BLV ELISA IgG interpretation using cutoff = 77800	Student's t-test two-tailed unequal variance
CMV pos n=10	186401	pos	0.045
CMV neg n=12	89113	pos	
HTLV pos n=11	171220	pos	0.132
HTLV neg n=12	122540	pos	
HIV pos n=12	290556	pos	0.006
HIV neg n=12	127389	pos	
HCV pos n=12	198735	pos	0.959
HCV neg n=12	194015	pos	
Hbsag pos n=12	299586	pos	0.771
Hbsag neg n=12	279105	pos	

**Table 2.** Results of testing sera positive and negative for IgG antibodies to CMV, HTLV, HIV, HCV, and Hbsag.

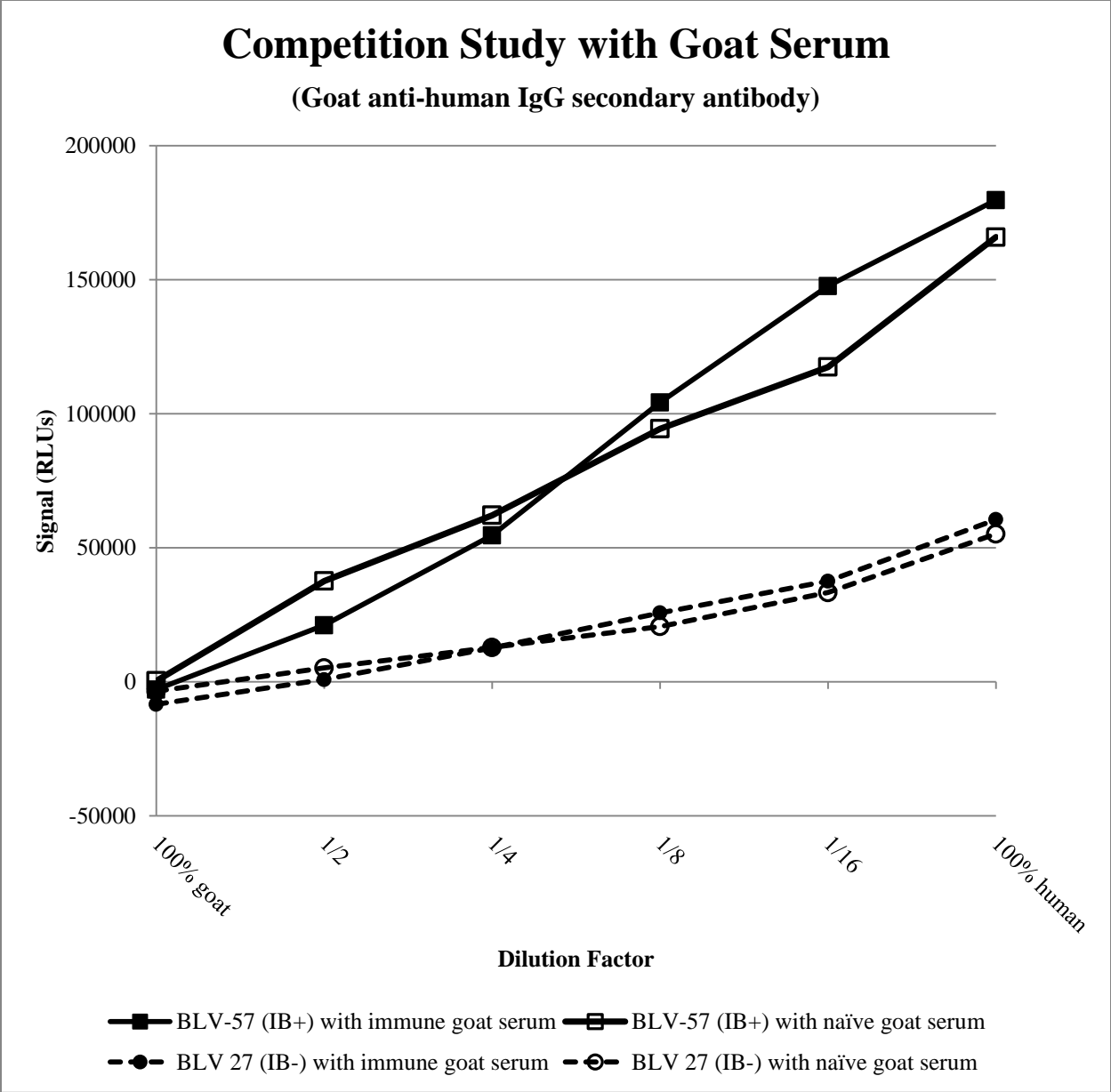


Figure 3a.

# Melon Gel Competition Study (Goat anti-human IgG secondary antibody)

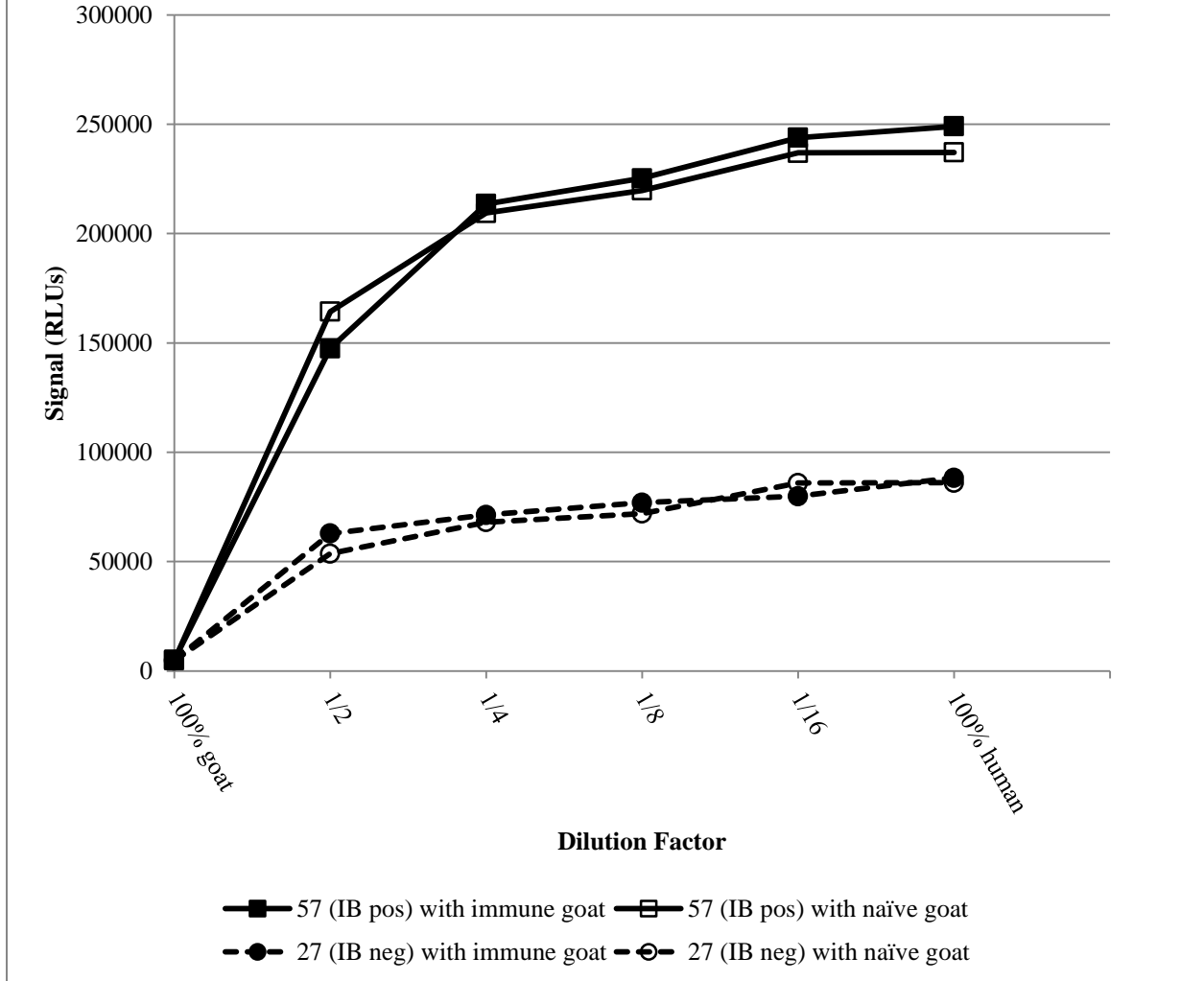


Figure 3b.

### Competition Study with Sheep Serum (Goat anti-human IgG secondary antibody)

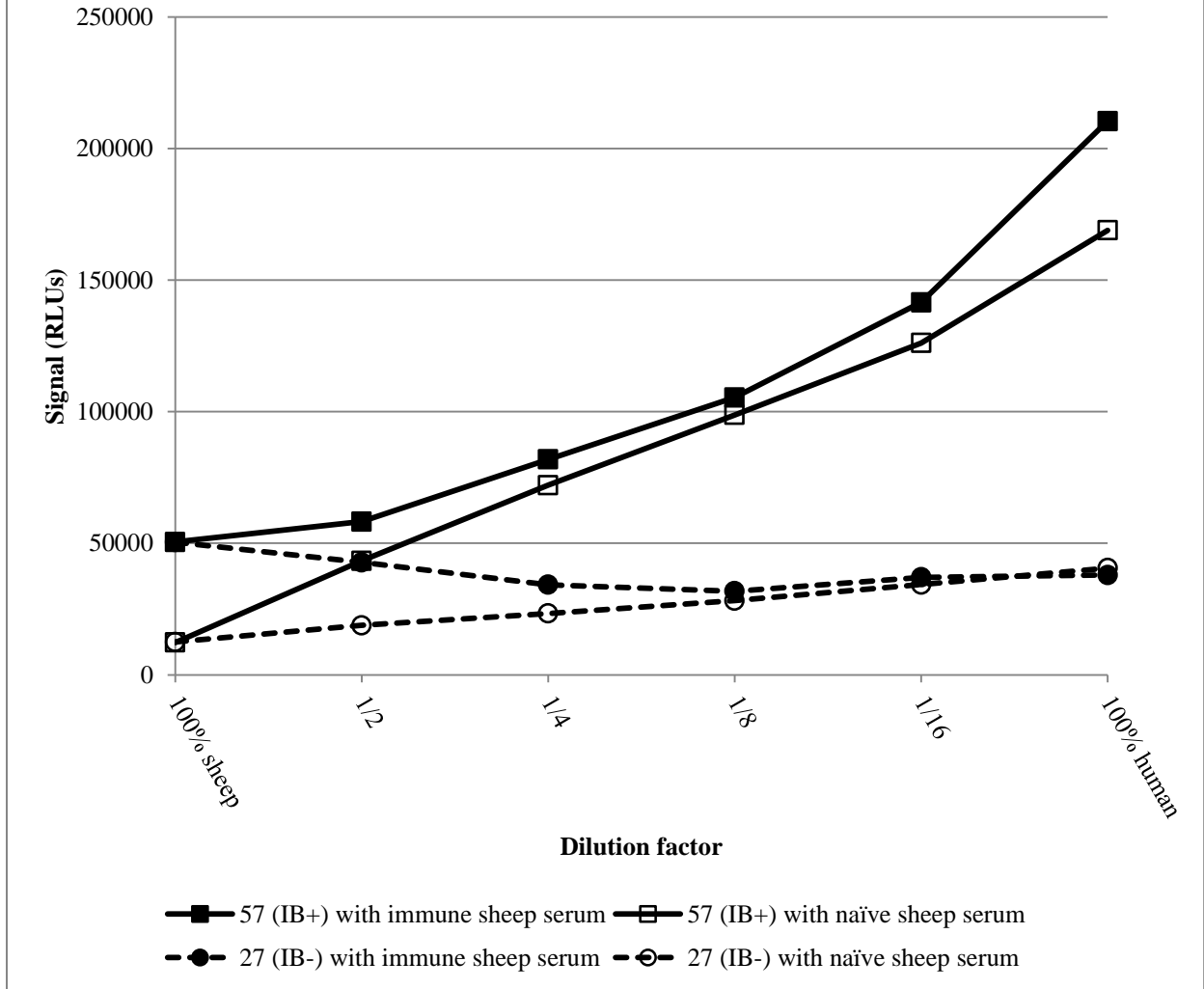
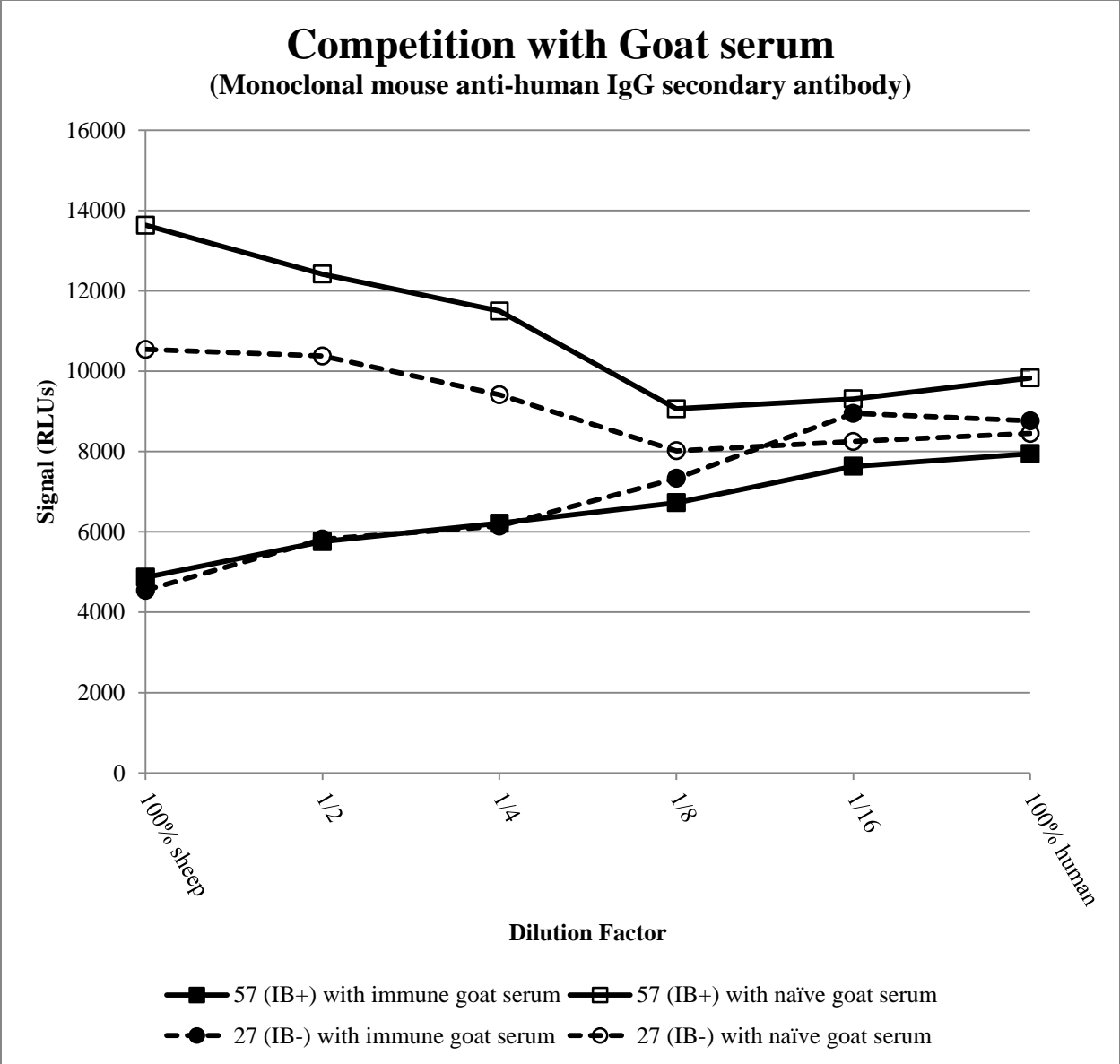
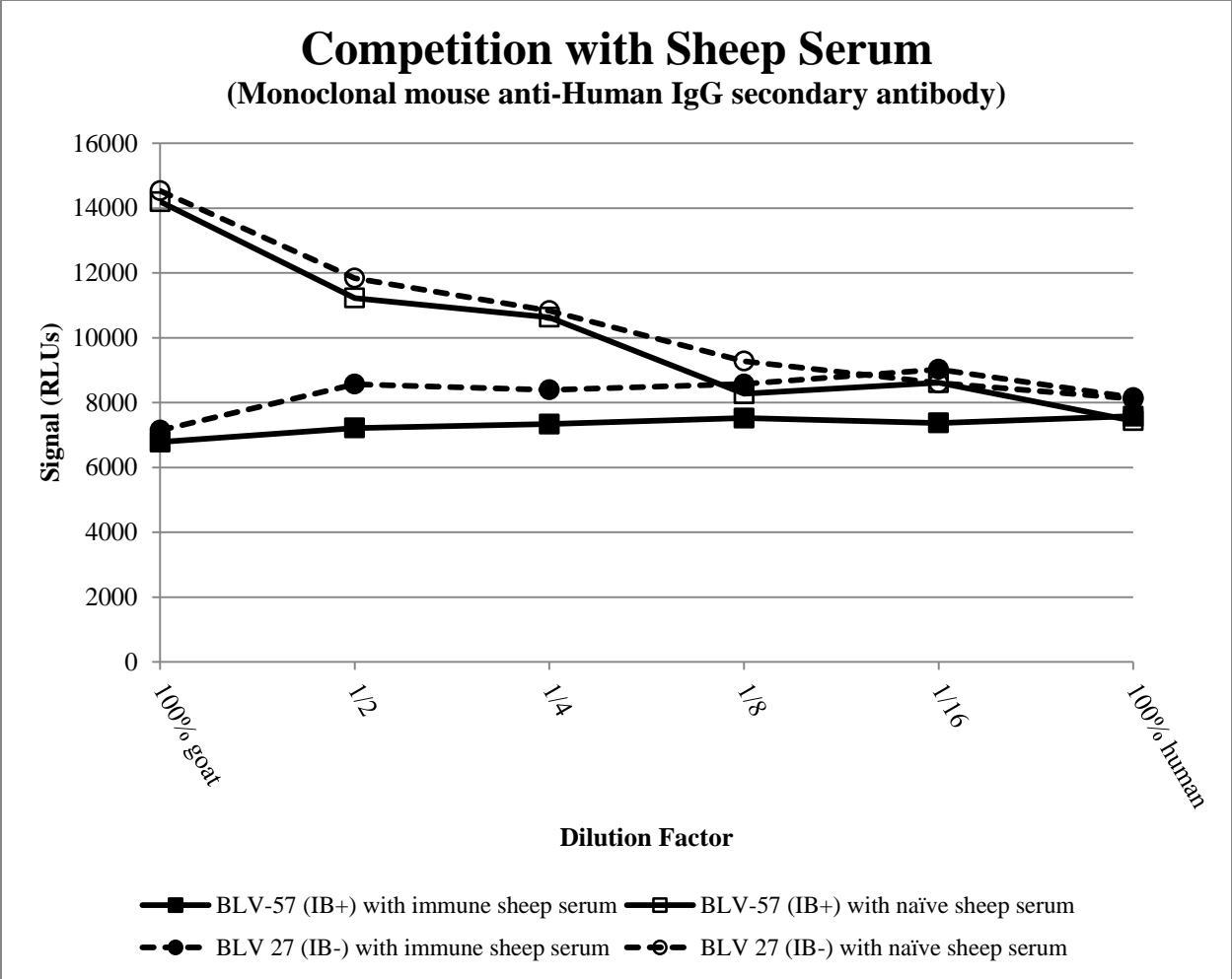


Figure 3c.



**Figure 3d.**



**Figure 3e.**

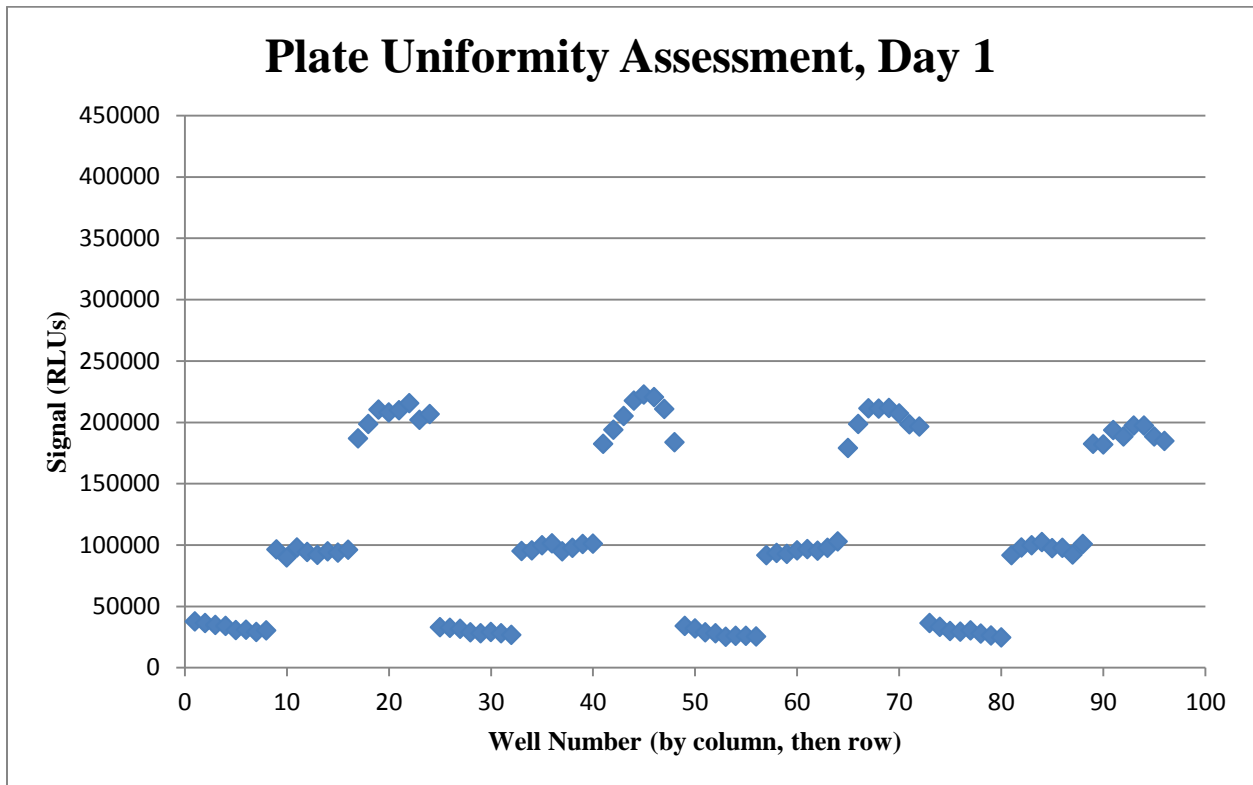
*Precision and Reproducibility*

Summary statistics for intra- and inter-plate reproducibility are presented in Table 3. The CV (coefficient of variation) was calculated by dividing the standard deviation (SD) by the square root of 2 (taking into account duplicate wells in the routine production assay), then dividing by the average RLU signal for those wells (AVG):  $CV = (SD/\sqrt{2})/AVG$ . No outliers were present. The signal window (SW) range, calculated for each day of reproducibility testing, was 17.4-24.3. Day 1 of the spatial uniformity assessment is displayed in Figure 4. Edge effects were most notable with the high sample on Day 1 (17.5%), with the outer wells having lower RLUs than the interior wells. Drift on the low signal ranged from 4.38%-9.91%; the medium signal drift ranged from -3.23%-7.98%; the high signal drift ranged from 2.64%-9.78%. All reproducibility testing parameters were within acceptable ranges, except for the intra-plate CV, which exceeded acceptable limits by 2.8%-4.3%.

<b>DAY 1</b>	<b>Min</b>	<b>Max</b>	<b>Mean</b>	<b>SD</b>	<b>CV (%)</b>
Low	24483	37658	29588	3520	8.4
Medium	89768	102840	96301	3433	2.5
High	179016	222558	199932	12462	4.4
<b>DAY 2</b>					
Low	21806	37149	25732	2873	7.9
Medium	79541	120212	104769	10701	7.2
High	189758	248990	226872	14068	4.4
<b>DAY 3</b>					
Low	48083	54294	51631	1871	2.6
Medium	175065	206784	190249	8477	3.2
High	341274	412544	384917	19693	3.6
<b>DAY 4</b>					
Low	39590	48537	44319	2274	3.6
Medium	143841	165350	153450	4774	2.2
High	192213	225596	207601	8699	3.0
<b>Inter-plate</b>					
Low	21806	54294	37818	12205	22.8
Medium	79541	206784	136192	43965	22.8
High	179016	412544	254831	87462	24.3

**Table 3.** Summary statistics, intra- and inter-plate reproducibility testing. Min=minimum signal for each sample (L, M, H) on the same plate, measured in relative light units (RLUs); Max=maximum signal for each sample (L, M, H) on the same plate, measured in relative light units (RLUs); mean= mean signal for each sample (L, M, H) on the same plate, measured in relative light units (RLUs); SD= standard deviation for each sample (L, M, H) on the same plate,

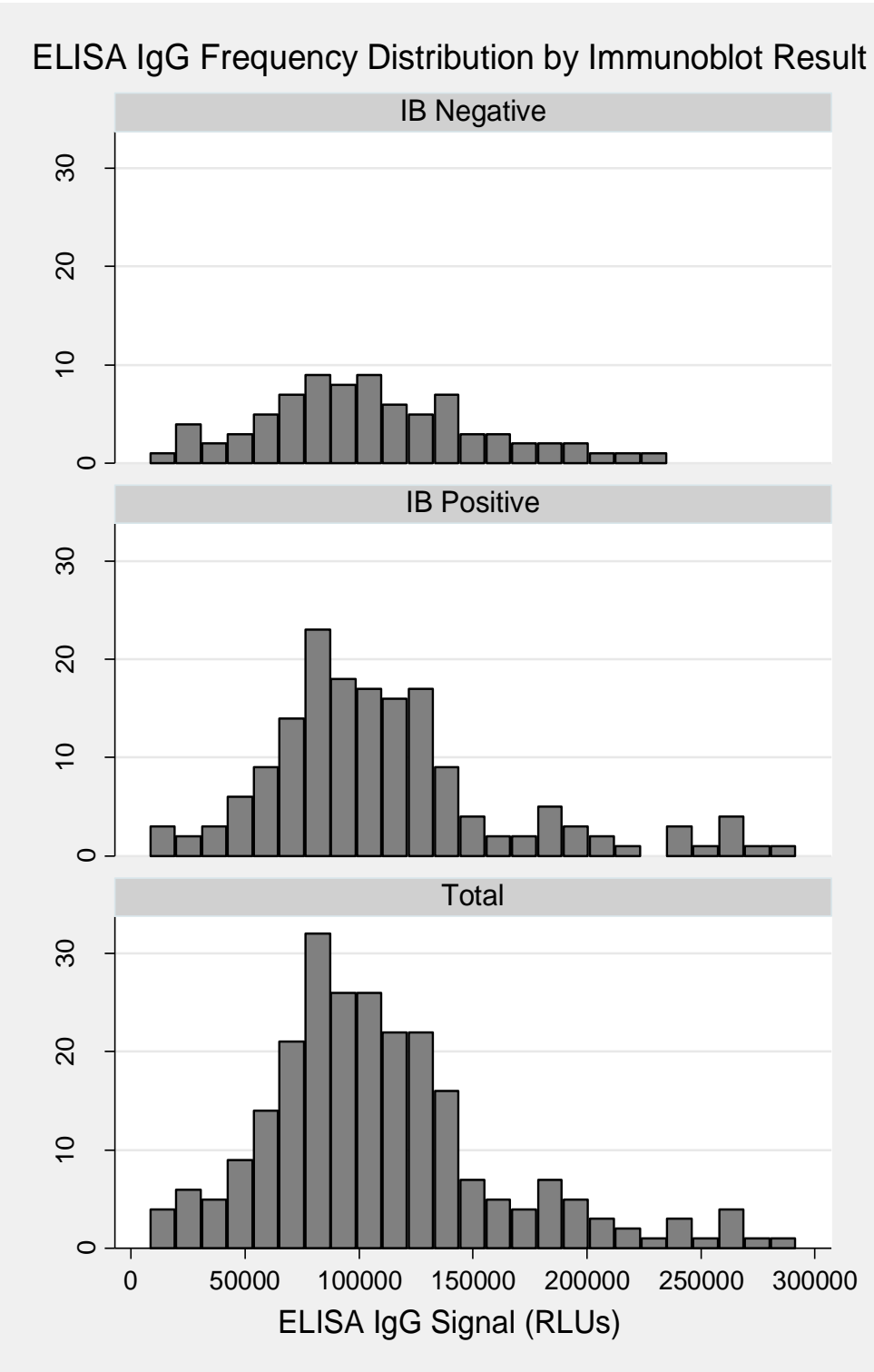
measured in relative light units (RLUs); CV (%) = percent coefficient of variation for each sample (L, M, H) on the same plate.



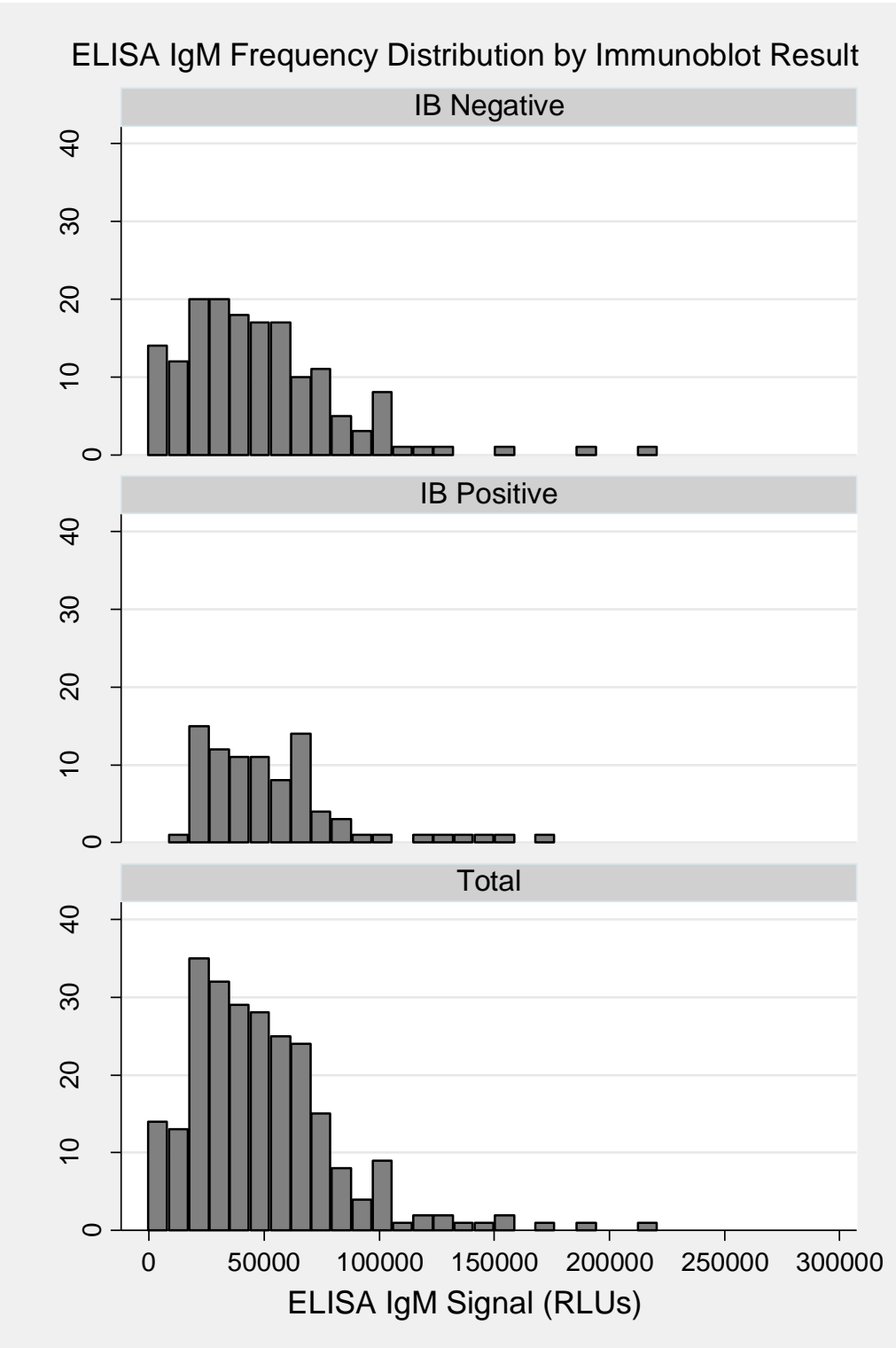
**Figure 4. Plate uniformity assessment, Day 1.** The horizontal axis indicates each microtiter well, number first by column and then by row. For example, well A1 is well #1; well H1 is well #8; and well A2 is well #9. Thus, each cluster of points reading from left to right on the horizontal axis represents the 8 wells of one column and proceeds through columns 1-12 of the microtiter plate. The vertical axis indicates the signal for that well measured in relative light units (RLUs).

#### ***Determination of the cut-off value***

Frequency histograms for IgG and IgM indicate a slightly right-skewed, unimodal, population (Figures 5 and 6). The two-sample Kolmogorov-Smirnov test (KS test) for equality of distribution functions was used to compare the ELISA signals of the immunoblot positive group to the immunoblot negative group. Although the immunoblot positive group did have a higher mean signal, there was no significant difference for IgG or IgM (Table 4).



**Figure 5.** ELISA IgG frequency distributions derived from the BLV-EPI study. Top, immunoblot (IB) negative group; middle, IB positive group; bottom, total study population (n=253).



**Figure 6.** ELISA IgM frequency distributions derived from the BLV-EPI study. Top, immunoblot (IB) negative group; middle, IB positive group; bottom, total study population (n=253).

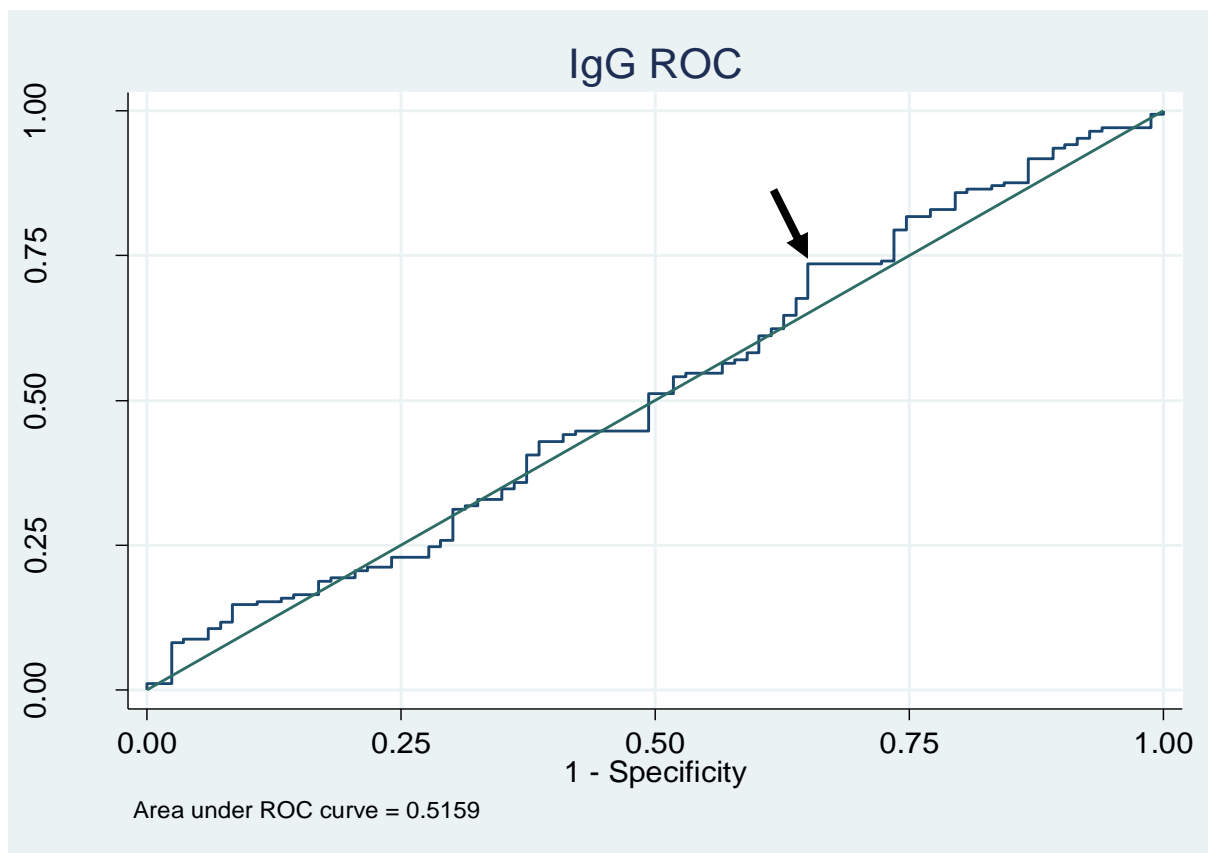
	ELISA IgG				ELISA IgM			
	Immunoblot positive	Immunoblot negative	KS test	Mann-Whitney	Immunoblot positive	Immunoblot negative	KS test	Mann-Whitney
number	n=170	n=83	0.78	0.68	n=89	n=164	0.13	0.10
Mean Signal (RLUs)	118730	112476			53335	47301		
95% CI	(108206.9-129253.1)	(98460.44-126491.5)			(46720.56-59949.19)	(42047.77-52555.04)		

**Table 4.** Mean ELISA IgG and IgM by immunoblot result. The two-sample Kolmogorov-Smirnov test (KS test) for equality of distribution functions, and the Mann-Whitney test, were used to compare the ELISA and immunoblot results.

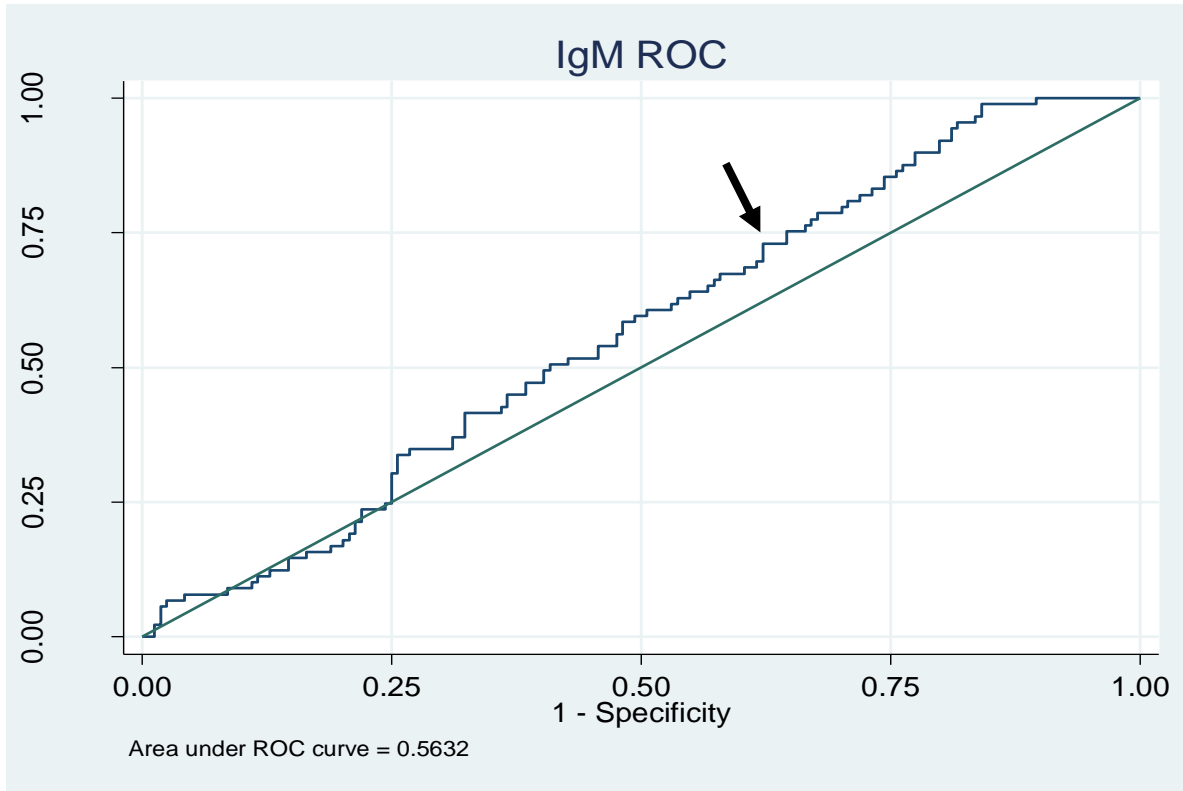
Clinical sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for representative centiles are listed in Table 5. Receiver-operator curve (ROC) discriminatory analysis using the samples' immunoblot results as the “gold standard” was performed to evaluate the performance of the ELISA assay and determine the optimal cut-off values (Figures 7 and 8). ROC analysis plots the true positive fraction (also known as sensitivity; y axis) against the false positive fraction (also known as 1-specificity; x axis) for every possible cut-off value. The closer the curve approaches the left-hand and top border of the graph, the more accurate the test; such a curve will have a higher area under the curve (AUC). The closer it approaches the 45° diagonal, the worse the accuracy. In general, an assay with an AUC of .9-1 is considered excellent; .8-.9 good; .7-.8 fair; .6-.7 poor; and .5-.6 fail. For this assay, the AUC for IgG is 51.6% (95% CI 0.454-0.581), while for IgM the AUC is 56.3% (95% CI 0.492-0.635), indicating that the assay performs inadequately compared with the immunoblot. The cut-off value selected for IgG is 77800 RLUs, which gives a sensitivity of 76.47% and a specificity of 26.1%. The cut-off value selected for IgM is 26244 RLUs, giving a sensitivity of 80.9% and a specificity of 28.05%. The effects of using different cut-off points on seroprevalence were modeled on the combined adult (BLV-EPI) and pediatric study populations (n=716) using the optimal cut-off described above, a cut-off with low sensitivity/high specificity, and a cut-off with high sensitivity/low specificity (Figures 9, 10, and 11).

IgG					IgM			
Percent positive	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Sens (%)	Spec(%)	PPV (%)	NPV (%)
90	91.8	13.3	68.4	44.0	98.9	14.6	38.6	96.0
80	81.8	22.9	68.5	38.0	86.5	23.8	38.1	76.4
70	71.8	34.9	69.3	37.7	75.3	33.5	38.1	71.4
60	58.8	39.8	66.7	32.0	64.0	43.3	38.0	72.8
50	48.8	50.6	66.9	32.6	53.9	53.0	38.4	68.0
40	39.4	62.7	68.4	33.5	44.9	63.4	40.0	68.0
30	30.0	69.9	67.1	32.8	33.7	73.2	40.1	67.0
20	20.6	79.5	67.3	32.8	18.0	79.9	32.7	64.2
10	11.8	91.6	74.1	33.6	9.0	90.1	34.8	64.8

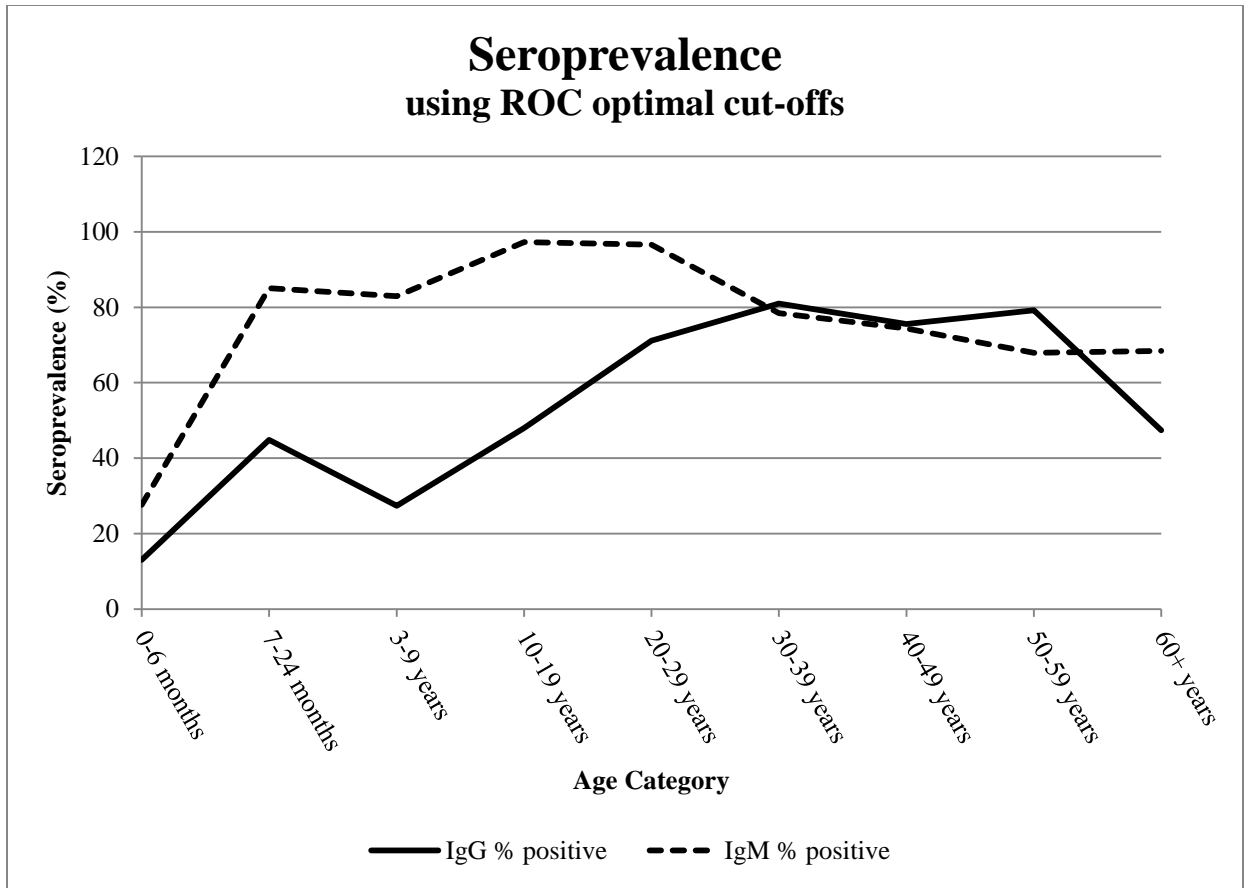
**Table 5.** ELISA IgG and IgM sensitivity (Sens), specificity (Spec), positive predictive value (PPV), and negative predictive value (NPV).



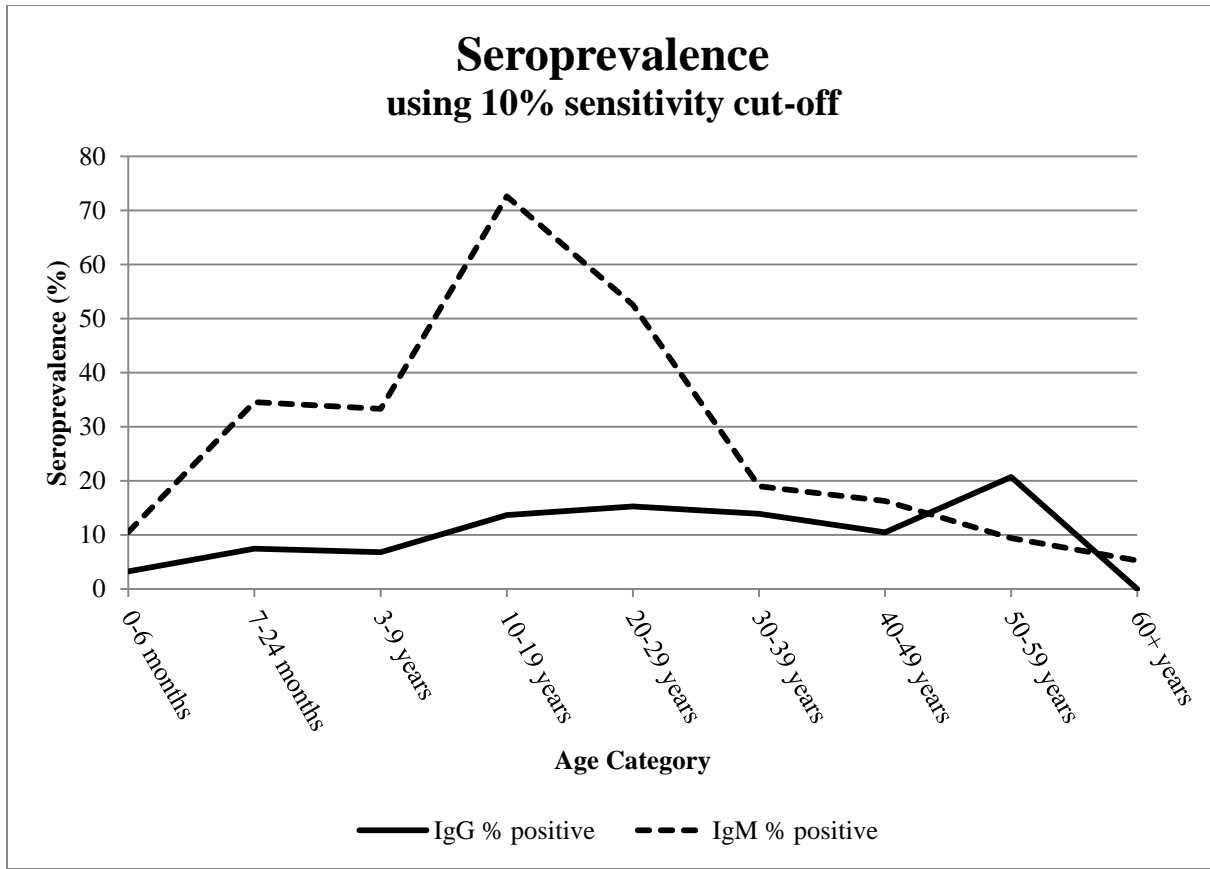
**Figure 7.** ELISA IgG receiver-operator curve (ROC) analysis, with immunoblot as the gold standard. The arrow indicates the point on the curve selected for the optimal cut-off value.



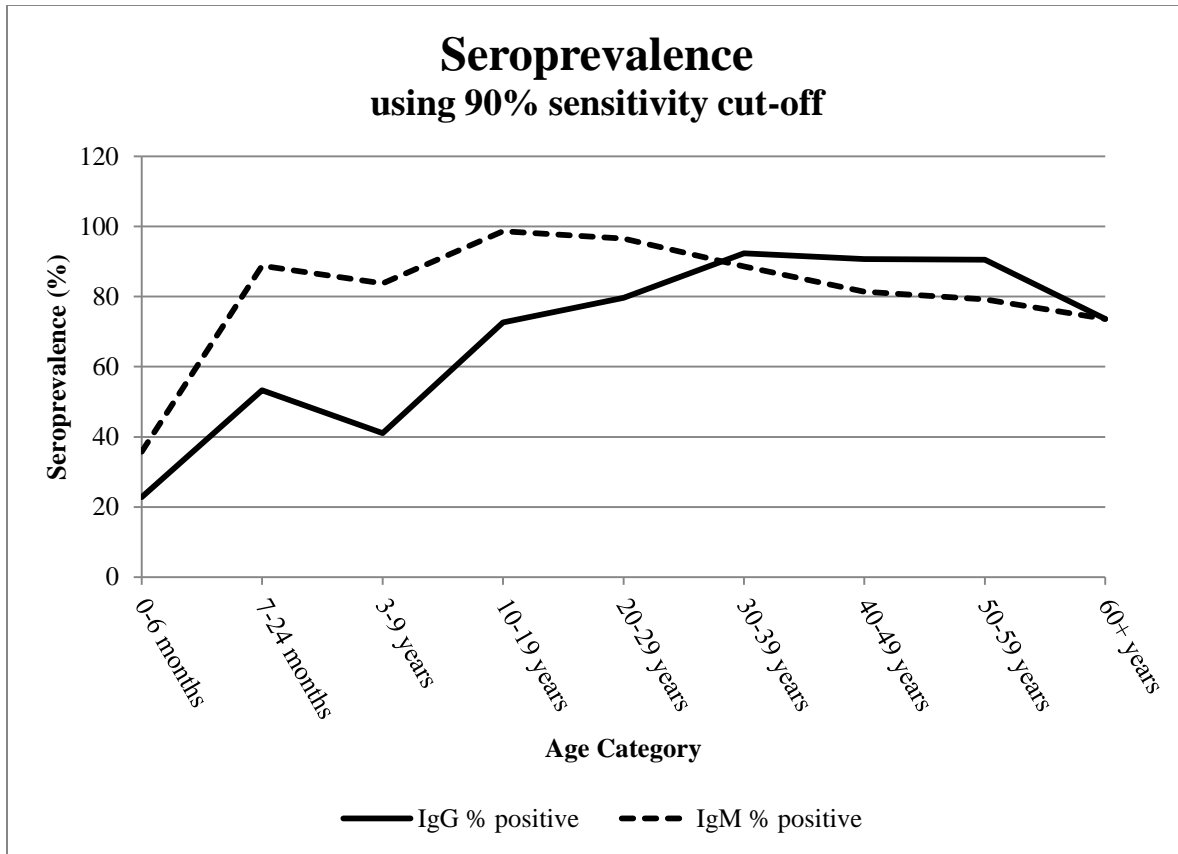
**Figure 8.** ELISA IgM receiver-operator curve (ROC) analysis, with immunoblot as the gold standard. The arrow indicates the point on the curve selected for the optimal cut-off value.



**Figure 9.** Seroprevalence of anti-BLVp24 antibodies derived from ROC analysis cut-off points. The overall seroprevalence pattern is consistent with the seroprevalence patterns of many other well-characterized infectious diseases. The more rapid rise of anti-BLVp24 IgM levels, followed by a lag in IgG production, is a typical immune response.



**Figure 10.** Seroprevalence of anti-BLVp24 IgG and IgM using a low sensitivity/high specificity cut-point. This approach yields a more conservative estimate of true BLV seroprevalence. The peak of IgM seropositivity in the 10-19 year old age group may be indicative of sexual transmission.



**Figure 11.** Seroprevalence of anti-BLVp24, using a high sensitivity/low specificity cut-point. This estimate has a high rate of false-positives, resulting in information of less utility to public health practice.

## Discussion

The human samples used for this assay development project were primarily archived serum samples obtained from the BLV immunoblot [5] study. Physical specimen integrity is crucial to the assay development process. To measure possible degradation of the samples during storage, three methods were used. First, human serum was electrophoresed and reacted with anti-human IgG, IgM, and IgA. Bands were present for all three isotypes, indicating that the immunoglobulin fraction was intact. Second, 10% of the archived sera were tested for anti-CMV IgG and anti-tetanus toxoid IgG and compared to the national CMV and anti-tetanus toxoid prevalences. The National Health and Nutrition Examination Survey III (NHANES III) took place in the United States from 1988-1994, close to the time period the samples for this study were collected, and is representative of the general United States population [44]. NHANES III data showed that CMV IgG prevalence ranged from 54.4-69.8% in persons 20-49 years of age [45]. Only 8% of the samples tested here was positive for CMV IgG, suggesting that the archived samples may have degraded to some degree. The difference in results between the two methods may be explained in part by the portion of the immunoglobulin molecule targeted. The anti-human antibodies used in the immunoblot procedure are specific only for the

Fc portion of immunoglobulin, while the CMV test measures functionality and specificity of both the Fc portion and the amino-variable end. Although the sample size for CMV testing was quite small and most likely not representative of either the study or the national population, the CMV test results may be more significant than the immunoblot test results, since it is the amino-variable end of the molecule that determines antibody specificity. As another, perhaps more reliable measure of sample integrity, IgG antibodies to tetanus toxoid were measured. Since the vast majority of the US population has been vaccinated with tetanus toxoid and kept up to date with periodic boosters, one would expect a positive level of anti-tetanus toxoid antibody in a high percentage of the samples if the integrity of the samples has been preserved. This was the case; 96% of the samples tested showed antibodies, higher than the 72.3% prevalence McQuillan et al [46] calculated using NHANES III data. McQuillan et al found that persons with more education were more likely to have protective tetanus-immune status than persons with less education. In this context, our data are valid, since the samples collected for this study were obtained from donors in a university community. Therefore, we conclude that the quality of the serum and plasma samples used in this study may be adequate for serology.

Immunoblots are labor intensive, time-consuming, and have a low throughput. In our lab, the BLV immunoblot procedure processes 20 samples (including controls) in two days. On a 96-well plate, allowing for appropriate controls and samples in duplicate, 41 samples can be tested in less than 8 hours. An indirect ELISA format was selected based on the intended purpose of the assay—to screen a large population for the presence of anti-BLV—and available reagents.

The checkerboard titration approach was employed to optimize this ELISA. Ideally, samples for assay development are selected using a blinded method. However, due to difficulty finding a concordant sample pair, pairs were chosen based on IB and ELISA results: a sample positive by IB and a high ELISA RLU, paired with an IB negative sample with a low ELISA RLU, were selected to conclude the optimization process. The coating buffer is a key component of ELISA, in that it promotes non-specific adhesion of the antigen to the microtiter plate surface. It is not surprising that a coating buffer with a higher pH improved the assay performance, since the pH of the coating buffer should be higher than the pKa of the protein antigen [47, 48] to allow optimal antigen coating.

Validation testing for specificity has mixed results. There was no significant difference in assay performance between serum or plasma samples, allowing samples from different study collections to be analyzed. In our experiment with a very small sample size, hemoglobin was shown to significantly decrease both IgG and IgM signals. However, we decided to include hemolyzed samples in the final analysis, since there was no significant difference between the mean IgM hemolyzed and non-hemolyzed signals in pediatrics aged less than one month (the age group with the highest proportion of hemolyzed samples), and the significant difference for the IgG signals only affects passively acquired maternal antibody.

Initial competition studies with a monoclonal anti-human secondary antibody indicates that the Assay may show specificity for the BLV p24 protein. In addition, there was no significant difference in the mean IgG signal of sera positive and negative for HTLV, HCV, Hbsag IgG antibodies, indicating that IgG antibodies to these viruses does not cross-react with the BLVp24 antigen. A significant difference was seen with HIV and CMV sera containing IgG to those viruses. This may be due to donors with dual HIV-CMV infection. A second explanation

involves the genetic relatedness of HIV and BLV. HIV, like BLV, is a retrovirus containing a p24 capsid antigen. Diagnostic serological tests for HIV include the p24 antigen, and confirmatory immunoblotting requires antibody to HIV p24 be present in order to be interpreted as positive. Although antibodies to HIV may be cross-reacting to the BLV p24 antigen, the prevalence of HIV positive donors in this study is most likely low and would potentially not affect estimates of BLV prevalence. CMV is a DNA virus in the Herpesviridae family. The HIV and CMV positive and negative test groups were all classified as BLV positive using a cut-off point of 77800RLUs, and any interference attributed to these cross-reactions may not be significant unless a higher cut-off point is employed for the BLV assay. Notably, antibodies to HTLV, the virus most closely related to BLV, did not cross-react with BLVp24. Melon™ Gel optimization experiments support the lack of specificity. The human SNR did not improve with sample clean-up, while the goat SNR was at least three times higher with Melon™ Gel clean-up than without it. However, sample clean-up with Melon™ Gel ruled out the possibility that interference from non-specific IgM (such as rheumatoid factor) was occurring, since most IgM is removed during the treatment process. In addition, no antigen control was available to rule out non-specific reactions with the antigen matrix, further reducing the validity of the ELISA specificity.

Testing of the inter- and intra-plate variability showed the assay to have excellent precision and reproducibility. The minor edge effects noted were most likely due to the delta time required to dispense the detection reagent, and was compensated for by off-setting the duplicates by one-half plate in either direction. Of some concern is the high inter-plate variation. Although the high signal had the highest variation, variation of the medium signal is more significant since it can affect whether a sample falls above or below the cut-off value. The slightly high inter-plate variation could be compensated for by increasing the number of replicates during routine testing and running them on separate days.

A variety of methods of determining an assay's cut-off value exist, including: 1) Empirical determination using a sample set of known negative sera from a "normal" population tested by the gold standard method. Two or three standard deviations above the mean of this pool is used as the cut-off value [49]; 2) Receiver-operator curve (ROC) analysis, where the true positives (sensitivity) are plotted against the false positives (1-specificity) to provide assay performance data over the entire assay range [50]; and 3) Local population prevalence and overall benefit to society [51]. A PubMed search was performed on 9/28/11 to determine which method(s) are most frequently used by researchers. The search parameter "'Enzyme-Linked Immunosorbent Assay/methods"[Mesh] cut-off value AND humans AND English" was entered and the first 50 accessible studies were used to tabulate the method for establishing the cut-off value. 18% used a sample set of known negatives, 52% used employed ROC analysis, and 4% used local population prevalence to set the cut-off value. As an example, in a study comparing *Helicobacter pylori* IgG by ELISA to histological staining of biopsy material, Gong et al (ref) found that the correlation between the two diagnostic methods was affected by smoking and gastric disease status. Therefore, the authors utilized ROC analysis to adjust the manufacturer's recommended ELISA cut-off value downward in order to improve the diagnostic capability of the ELISA in certain populations. In addition, 14% used the manufacturer's recommended cut-off values, while one statistical theory paper discussing a diagnostic method using predictive probabilities without cut-offs, and one paper using a tree-regression method were found.

ROC analysis was used to select the cut-off values here because of its inherent advantages. All possible cut-points were used to develop a visual representation of assay performance, clearly showing the trade-off between assay sensitivity and specificity. The area under the curve (AUC) is a measure of the assay's accuracy, or ability to discriminate between two groups. An area of 1 represents a perfect test, with a curve following the left and then upper border of the graph. An area of 0.5 represents a worthless test; in this case the curve will be close to the diagonal line. The ELISA method developed here has poor ability (IgG AUC=0.51, IgM AUC= 0.56) to discriminate between people with and without antibodies to BLV p24<sup>gag</sup>, compared to immunoblot. ROC analysis is concordant with the unimodal IgG and IgM frequency distributions.

Inclusion of a surface envelope protein may improve assay performance. Many veterinary/agricultural BLV immunoassays utilize the gp51 protein in conjunction with p24. However, this protein is less sensitive than p24, possibly due to denaturation during the heating and electrophoresis stages of immunoblotting (reviewed in [52]). Similarly, for HTLV it was shown that immunoblot is more sensitive to *gag* proteins, while radioimmunoprecipitation assay (RIPA) is more sensitive to *env* proteins, presumably due to methodological differences [53].

Although gp51 may be suitable for ELISA, which does not denature the antigen, we chose to use only a recombinant BLV p24 protein, since BLV group specific antigen p24 (p24<sup>gag</sup>) is the major structural protein and is expressed in higher quantity than other viral proteins. Viral surface proteins of cell-associated viruses such as BLV and HTLV are produced in limited quantity and, if prepared from whole viral lysate, tend to degrade more rapidly than p24<sup>gag</sup> [52].

Contrary to the above logic regarding levels of expressed *env* protein, inclusion of BLV surface antigens may improve the performance of the ELISA assay. Examining the history of assay development with HTLV, the virus most closely related to BLV, may provide the basis to support inclusion of surface proteins in ELISA. HTLV produces few surface envelope proteins (*env*) with low levels of expression on host cell surfaces [54] yet there is a clear humoral response to these *env* proteins. Due to low amounts of *env* proteins in native viral lysates, HTLV assays evolved to include recombinant *env* proteins [54, 55]. One recombinant *env* protein (r21e) significantly increased the HTLV assay sensitivity but also increased false-positives [56-58]. Later, an alternative recombinant, gd21, maintained a high sensitivity while improving specificity [55]. Therefore, it is reasonable to include BLV *env* in future ELISA development projects.

Assay performance is also dependent on the prevalence of antibodies to that antigen in the population being tested. For instance, a common problem with interpretation of HTLV immunoblot results is the occurrence of *gag* indeterminate profiles, where antibodies to p24 are lacking. Immunoblot profiles with reactivity only to envelope proteins are less common [59]. Whether such indeterminate antibody profiles occur with human BLV infection will only be elucidated with further research.

In addition, for optimal comparison of this ELISA with the IB method, both methods should be run concurrently on newly collected specimens. Concurrent testing will remove any concerns regarding physical specimen integrity due to storage issues. To improve the specificity of this assay, further development is required. For instance, human anti-BLV can be purified by using an amino link column to which p24 is bound. This purified human anti-BLV can be

spiked into immunoglobulin depleted human serum to determine minimum detection limits, the linear range, construct a calibration curve, and used as a positive control on routine assay runs.

In conclusion, although this ELISA shows excellent reproducibility, it may lack sufficient specificity, as there is poor correlation with the immunoblot results from the archived samples, no immunoblotting was performed on the newly collected specimens to confirm whether the ELISA results, and no antigen control was employed. However, competition studies with the monoclonal anti-human secondary antibody may confirm specificity. Keeping in mind that BLV assay development for human purposes is in its initial stages, and that with further development, the ELISA method could be employed instead of the immunoblot method to save time and resources during future seroepidemiological studies.

## **Chapter 3: Seroprevalence of Bovine Leukemia Virus Antibodies in Humans**

### **Introduction**

Seroprevalence studies using age-related patterns of agent-specific antibody prevalence are frequently employed to identify populations at risk, evaluate immunization programs, and define transmission routes. Other population characteristics such as socioeconomic status and race/ethnicity can also provide useful information for public health practice. For example, a study of herpes simplex type 2 (HSV-2) in three Korean populations showed that the rate of HSV-2 was much higher in commercial sex workers and in HIV infected men than in the general public. HSV-2 seroprevalence in the Korean general public increases significantly beginning with the 30-year age group [60]. These data point to the sexual transmission route of HSV-2, and identifies demographics that should be targeted with prevention and control programs. In a study of two groups of African children, two transmission patterns for Kaposi-sarcoma associated herpes virus (KSHV) were identified. In South African children, no age-dependency of KSHV antibodies was present, while in the Ugandan group a significant age-dependency pattern was noted. Because KSHV seroprevalence was less than 10% in 1.5 year-olds, and steadily increased to nearly 40% in 8 year-olds, the authors concluded that transmission in the Ugandan children was primarily horizontal and non-sexual [61], perhaps through saliva. The authors were able to exclude sexual transmission because the study populations did not include age groups where sexual activity occurs. In another seroprevalence study, Dollard et al [45] investigated the relationship between cytomegalovirus (CMV) IgM and IgG titers and avidity to improve detection primary CMV infection, thus positively impacting management of pregnancy and treatment options for the fetus.

The seroprevalence patterns of HTLV-1, as the virus most closely related to bovine leukemia virus (BLV), may serve as a model for BLV seroprevalence. Furnia et al [62] were able to determine the time frame for transmission of HTLV-1 to infants during the breast-feeding period by testing for specific IgG and IgM. In infants less than 6 months old, IgG but not IgM was present, suggesting the IgG antibodies were maternal antibodies that had crossed the placenta. In infants greater than 6 months old, both IgG and IgM was detected, which suggested that the infants had become infected and were producing IgM as their primary immunologic response. The data allowed physicians to recommend breast-feeding up to 6 months of age without significant risk of HTLV-1 transmission, which may be preferable to alternative adverse outcomes associated with the lack of breast-feeding. HTLV-1 seroprevalence rises consistently with age. In persons under 20 years of age, the rate of positivity between males and females is similar. In women, seropositivity continues to increase, while in men levels plateau; thus, females over the age of 50 years have a significantly higher seroprevalence than men of the same age. This phenomenon may be the result of cumulative vertical, sexual, and blood exposures [63].

BLV seroprevalence studies have been initiated (Buehring et al., unpublished data). The age-dependency of anti-BLVp24 was analyzed in a sample of 107 individuals using gel blot and dot blot assays. Children under 6 months of age had only IgG antibodies, most likely maternal antibodies that crossed the placenta, while individuals 6 months and older also had IgM (12% frequency, overall) and IgA (23% frequency, overall). The sample size was too small to

achieve a representative frequency of age distribution, and therefore could not identify a definitive seroprevalence pattern. These results suggested that exposure to BLV may occur at a very young age, consistent with the hypothesis that transmission to humans occurs via dairy consumption or breastfeeding.

The research presented here expands on the preliminary serological studies by increasing the sample size of pediatric donors and employing the preliminary ELISA method described here (Chapter 2) to detect anti-BLVp24 IgG and IgM, with the intent of estimating the age at which humans become infected with BLV. The following data are estimates based on this first generation ELISA, the specificity of which has not been supported by competition studies or antigen controls.

## Results

### *Study Population Characteristics*

A total of 732 donor samples from two study population were initially available, of which 447 were newly collected pediatric samples, while 285 were archived samples from the previous study [5]. After excluding donors with missing data or sera, 707 samples were eligible for inclusion. Donor age ranged from 1 hour to 80 years, with a median age of 10.5 years. Gender distribution was unequal, with 35.6% males and 64.4% females overall. Females predominate in the adult study population (Table 6).

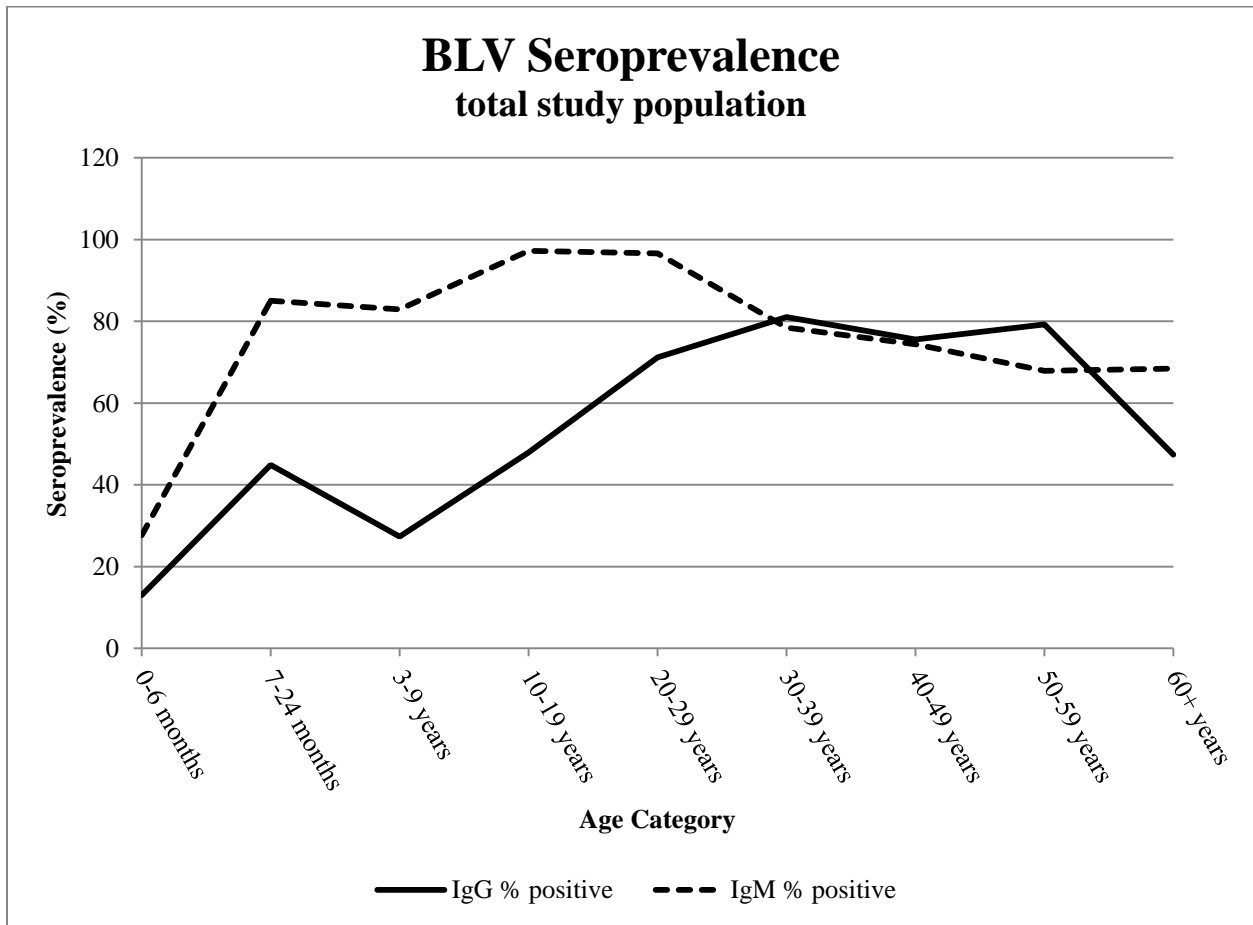
Age Category	Sex		Total
	Female	Male	
0-6 months	59	64	123
7-24 months	45	53	107
3-9 years	50	67	117
10-19 years	43	30	73
20-29 years	50	9	59
30-39 years	66	13	79
40-49 years	78	8	86
50-59 years	48	5	53
60+ years	16	3	19
Total	455	252	707

**Table 6.** General characteristics of study populations.

### *BLV seroprevalence*

Of the total study population, 49.3% were IgG positive, while 73.3% were IgM positive. In donors 20 years of age and older, 78.4% are IgM positive and 75.0% are IgG positive. In children aged 0-24 months, 27.8% were IgG positive, while 54.4% were IgM positive. Figure 12 depicts anti-BLVp24 IgG and IgM seroprevalence in the total study population. Levels of both

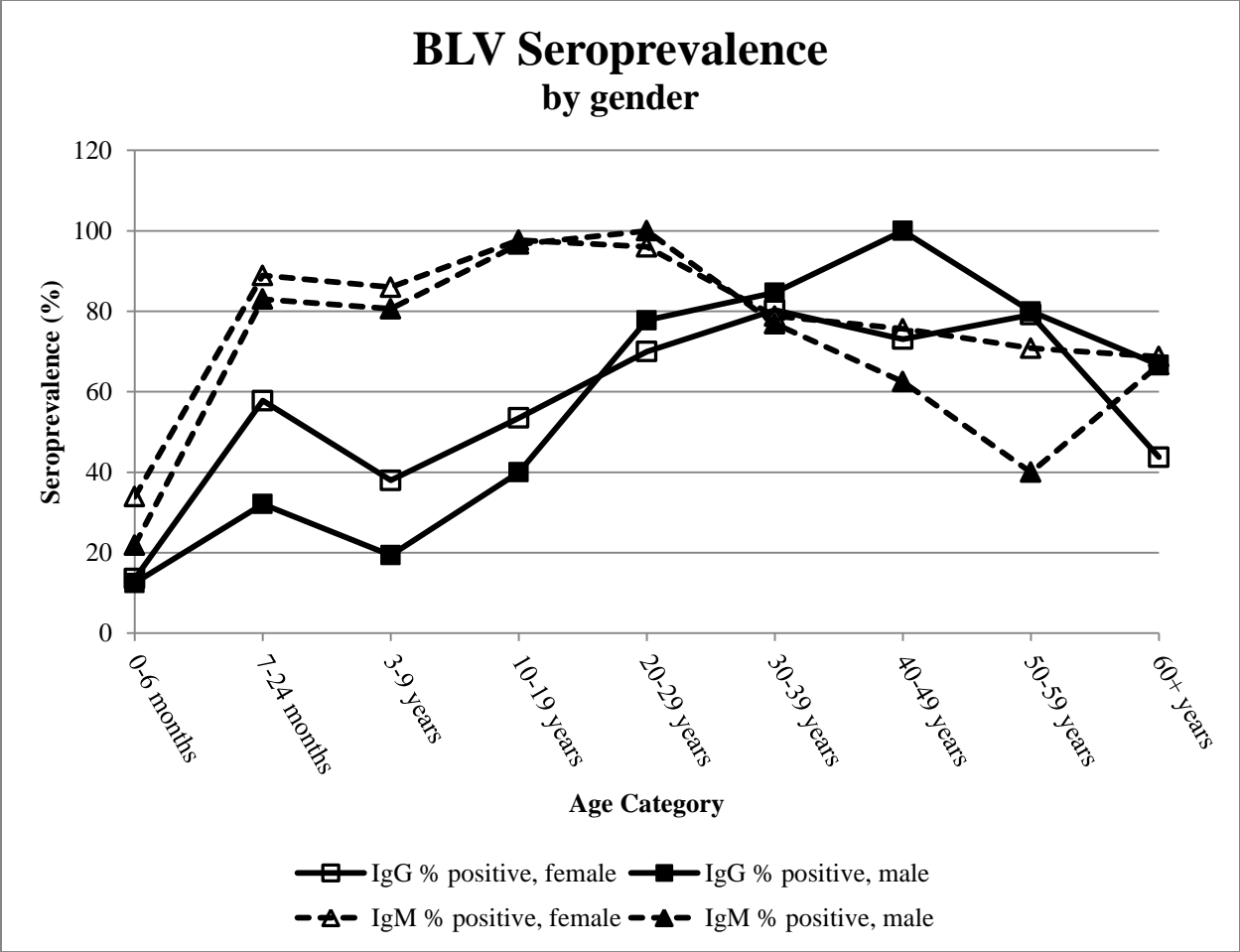
antibodies rise significantly from 0-24 months of age, with IgM peaking in the 10-19 year age range and IgG peaking in the 30-39 year age range (Table 7). Males and females generally have a similar seroprevalence pattern (Figure 13), although older females may have higher IgM seropositivity than males of the same age. Pediatric donors aged less than two years are more closely examined in Figure 14 and Table 8. IgG is present in 14.2% of 0-3 months olds, declining to non-detectable levels by 6 months of age. IgG seroprevalence then begin to increase until the 16-18 month age group. Remarkably, IgM is present in 23.9% of newborns, rising to 70.0% by 6 months of age. IgM seropositivity is consistently higher than IgG seropositivity during the first two years of life.



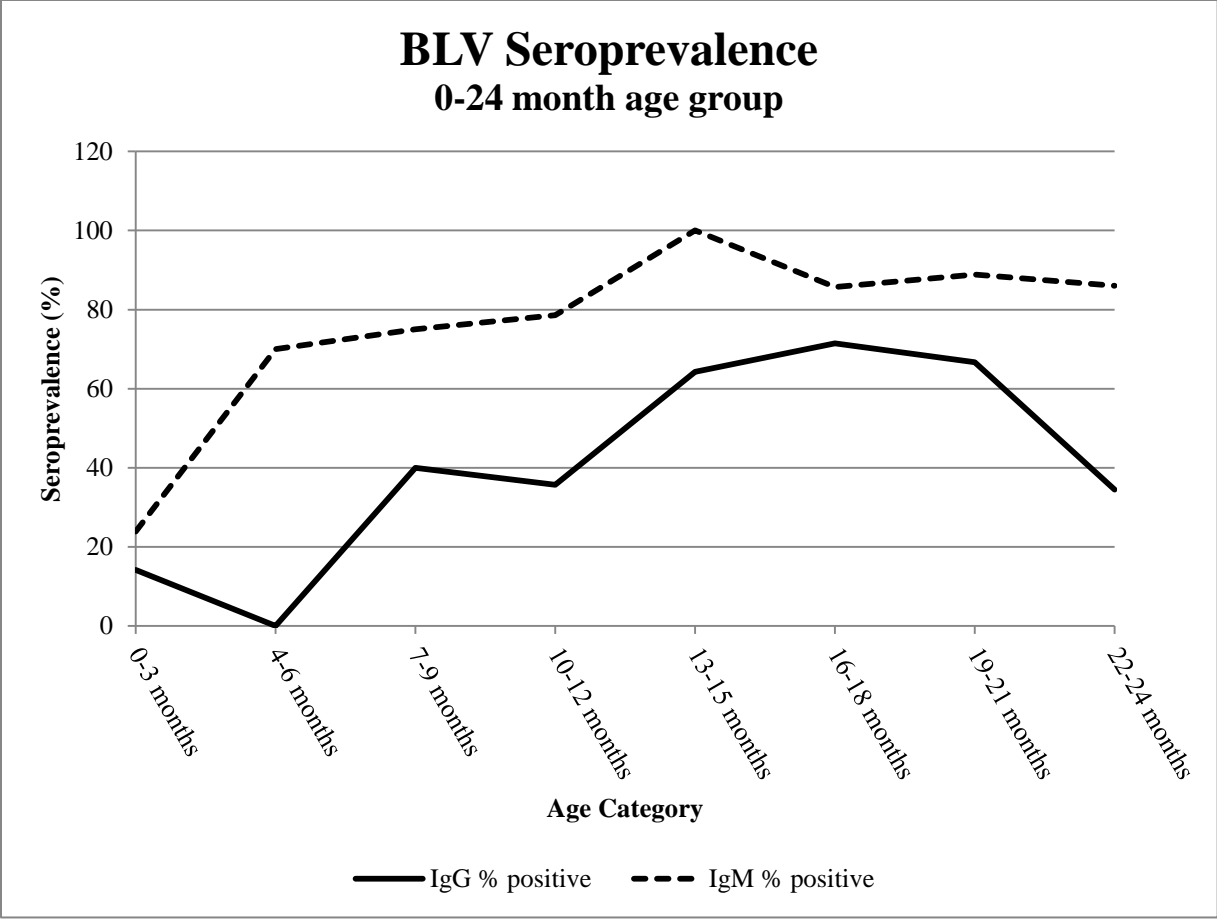
**Figure 12.** Anti-BLVp24 IgG and IgM seropositivity in the total study population.

Age Category	IgG proportion positive				IgM proportion positive			
	Female n=455	Male n=252	Total n=707	P <sub>trend</sub>	Female	Male	Total	P <sub>trend</sub>
0-6 mos n=123	0.136	0.125	0.130	--	0.339	0.219	0.276	--
7-24 mos n=107	0.578	0.321	0.449	0.000	0.889	0.830	0.851	0.000
3-9 yrs n=117	0.380	0.194	0.274	0.006	0.860	0.806	0.829	0.664
10-19 yrs n=73	0.535	0.400	0.480	0.004	0.977	0.967	0.973	0.003
20-29 yrs n=59	0.700	0.778	0.720	0.007	0.960	1.000	0.966	0.829
30-39 yrs n=79	0.803	0.846	0.810	0.178	0.788	0.769	0.785	0.002
40-49 yrs n=86	0.731	1.000	0.756	0.400	0.756	0.625	0.744	0.541
50-59 yrs n=53	0.792	0.800	0.793	0.617	0.708	0.400	0.679	0.410
60+ yrs n=19	0.438	0.667	0.474	0.009	0.688	0.667	0.684	0.968
Totals	0.585	0.325	0.493	0.000	0.767	0.671	0.733	0.000

**Table 7.** Proportion of IgG and IgM seropositivity in the total study population, by age and gender. P<sub>trend</sub> is the p value of the non-parametric test for trend across ordered groups. The P<sub>trend</sub> values apply to the combined male and female donors for that age category, compared to the next younger age category. A value <0.05 is considered significant.



**Figure 13.** BLV seroprevalence in the total study population, by gender.



**Figure 14.** Anti-BLVp24 IgG and IgM seropositivity rates for donors aged less than two years.

Age Category (months)	IgG proportion positive				IgM proportion positive			
	Female n=104	Male n=117	Total n=221	P <sub>trend</sub>	Female n=104	Male n=117	Total n=221	P <sub>trend</sub>
0-3 n=113	0.151	0.133	0.142	--	0.321	0.167	0.239	--
4-6 n=10	0	0	0	0.204	0.500	1.000	0.700	0.002
7-9 n=20	0.500	0.357	0.400	0.022	0.750	0.786	0.750	0.774
10-12 n=14	0.667	0.222	0.357	0.803	1.000	0.778	0.786	0.812
13-15 n=14	0.667	0.714	0.643	0.138	1.000	1.000	1.000	0.072
16-18 n=7	0.600	0.600	0.714	0.749	0.800	0.800	0.857	0.157
19-21 n=9	0.667	0.600	0.667	0.844	0.667	1.000	0.889	0.854
22-24 n=43	0.542	0.111	0.345	0.080	0.917	0.778	0.861	0.822
Totals	0.327	0.214	0.278	0.000	0.577	0.496	0.544	0.000

**Table 8.** Proportion of IgG and IgM seropositivity in the 0-24 month age group, by age and gender. P<sub>trend</sub> is the p value of the non-parametric test for trend across ordered groups. The P<sub>trend</sub> values apply to the combined male and female donors for that age category, compared to the next younger age category. A value <0.05 is considered significant.

## Discussion

In this first seroprevalence study of antibodies to bovine leukemia virus in humans, the overall seroprevalence pattern is consistent with the seroprevalence patterns of many other well-characterized infectious diseases [64]. The earlier rise of anti-BLVp24 IgM levels, followed by a lag in IgG production, is a typical immune response. The relatively high overall percentage of IgM seropositivity (73.3%) compared to IgG seropositivity (49.3%) may be due to the low median age of the study population, since IgG production lags behind IgM production. If donors less than 20 years are excluded from analysis, this gap is not evident. The level of IgG peaks at 30-39 years of age, plateaus, and then begins to decrease at 60 years of age. IgM peaks in adolescence and then begins a slow decline after 30 years of age, but never falls below 60% prevalence. Continuing high IgM titers may be indicative of constant reexposure to BLV via dairy and meat consumption, or to episodes of viral reactivation [65].

Estimates of antibody frequencies may indicate the occurrence of vertical transmission. Low levels of IgG antibody are present in the 0-3month age group, declining to undetectable

levels by the age of six months. This IgG is most likely maternal IgG that crossed the placenta and then waned below levels of detection by the age of 6 months. Unlike the seroprevalence pattern of HTLV-1, in which no IgM antibody is detectable until after the age of 6 months [62], in this study 23.9% of 0-3 month olds are IgM positive, increasing to 70% IgM seropositivity by the age of 6 months. Like HTLV-1, BLV is highly cell-associated with low levels of viremia, therefore it is unlikely that transmission occurs in utero. BLV transmission could have occurred during the trauma of birth, when the likelihood of infected lymphocyte transfer from mother to infant is higher, or after birth via breastfeeding. Prospective studies of mothers and infants that follow serostatus and viral load status of the study participants will be needed to establish temporal relationships in BLV vertical transmission. It may be that the maternal anti-BLVp24 IgG does not confer protection to the infant. Increasing IgM seropositivity from 4-6months of age to 22-24 months of age, plateauing through the 3-9 year old age group, may be indicative of horizontal non-sexual BLV infection acquisition during the weaning period (through 24 months old), as infants are exposed to dairy products. IgM seropositivity increases again from the 3-9 year old age group, achieving peak levels in the 10-19 year old group, potentially indicative of sexual transmission. In addition, when examining the seroprevalence of IgG and IgM by gender, the seropositivity rates are similar between males and females until 39 years of age, when there is some divergence between the two groups. Older females appear to have a higher IgM seropositivity than males, while for IgG the opposite is true. This pattern is somewhat similar to the seroprevalence pattern of sexual transmission of HTLV-1 discussed earlier. However, our data include limited numbers of males over 20 years of age, and further studies will need to confirm sexual transmission.

## Concluding Remarks

Widespread consumption of BLV-contaminated dairy and beef may potentially be responsible for a significant proportion of breast cancer cases worldwide. If the pathogenicity of BLV for humans is established, the implications are far-reaching. Using the preliminary ELISA method being developed here, this study may indicate that both vertical and sexual transmission of BLV potentially occur. However, presence of antibodies to the BLV p24<sup>gag</sup> protein do not necessarily indicate infection, but may result from exposure to the antigen from consumption of dairy products. In addition, confirmation of the ELISA specificity is necessary to make firm conclusions. Further prospective studies are warranted in order to determine precisely at what point(s) in the perinatal period vertical transmission could occur. Prospective studies may also permit a study sample more representative of the target population to be developed. More precise estimates of seroprevalence can help elucidate whether sexual transmission is truly occurring.

In the future, primary preventative measures should be taken to avert continuing infection of humans. Such measures might include a national level control and eradication program to ensure a safe food supply, even though it may take decades to see results. The costs involved in eradicating BLV from US herds might be offset by eventual recuperation of losses due to culling of infected animals and inability to export products to certain foreign markets. Early detection screening tests for humans could be developed, but would be useful only if effective treatments were available. If mother-to-child transmission via breast milk were confirmed to occur, early detection of BLV infection would enable breast-feeding mothers to avoid transmitting the virus to their infants. Establishing causality and determining transmission routes is critical for reducing the significant morbidity and mortality burden due to breast cancer.

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