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An exploration of amniotic fluids as a possible source of fetal infection in the feline immunodeficiency virus (FIV)-infected cat model of pediatric aid

Brittany Tenille Clay

AN EXPLORATION OF AMNIOTIC FLUIDS AS A POSSIBLE SOURCE OF FETAL INFECTION IN THE FELINE IMMUNODEFICIENCY VIRUS (FIV)-INFECTED CAT MODEL OF PEDIATRIC AIDS

By

Brittany Tenille Clay

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By

Brittany Tenille Clay

Approved:

Karen S. Coats Associate Professor of Biological Sciences (Major Professor)	Dwayne A. Wise Professor of Biological Sciences (Committee Member)
Gregory T. Pharr Associate Professor Veterinary Medicine (Committee Member)	Gary Ervin Associate Professor of Biological Sciences (Coordinator of Graduate Studies)

Gary Myers
Dean of the College of Arts and Sciences

Name: Brittany Tenille Clay

Date of Degree: May 1, 2010

Institution: Mississippi State University

Major Field: Biological Sciences

Major Professor: Dr. Karen S. Coats

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Candidate for Degree of Master of Science

The role that amniotic fluid (AF) may play in HIV vertical infection is unresolved. We used the FIV-infected cat model to study this question. We hypothesized that AF may be a source of fetal infection if the virus is present in the fluids. However, virus neutralizing (VN) antibodies in AF may limit vertical transfer. Fetuses were delivered from FIV-infected queens by cesarean section at early and late gestation. AFs were aspirated from intact fetal membranes and tested for viral antigen and RNA and for FIV-specific antibody. Randomly-selected samples were tested for VN activity using a syncytium reduction assay. Neither FIV antigen nor RNA was detected in any AFs. AFs and parallel serum samples from early and late pregnancy were positive for FIV-specific antibody. VN activity was detected in three early-term AFs and a parallel serum, but not late-term AFs. AF appears to play no appreciable role in FIV vertical transmission.

DEDICATION

I would like to dedicate this work to one of my best friends, Demarcus Antonio Travis. I hope that my hard work, determination, and sacrifice during my years as a mastered student will inspire him to follow his dreams and pursue all opportunities in spite of any hardships.

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CHAPTER I

REVIEW OF PERTINENT LITERATURE

Primary infection by FIV is marked by an acute phase, lasting 1-4 weeks. This phase may be clinically silent in some individuals and/or may be manifested as depression, anorexia, neutropenia, and other symptoms. The acute phase can lead to major life-threatening bacterial infections. The acute phase is followed by the asymptomatic phase in which symptoms are absent and the virus may appear to have completely disappeared, since the infection sometimes remains silent for years. Some accompanying symptoms are depression, anorexia, acute diarrhea, dermatitis, gingivitis, and upper respiratory problems. The persistent generalized lymphadenopathy phase is characterized by swelling of the cat's lymph nodes and infrequent, very vague symptoms, including fever, anorexia, weight loss, and sometimes nonspecific behavioral changes. During the AIDS-related complex (ARC) phase, the infected cats show chronic secondary infections of the oral cavity, upper respiratory tract, and other body sites. These infections are usually caused by various pathogens including *Pseudomonas sp., Microsporum canis, and Steptococcus canis*. Eventually, infected cats progress to feline AIDS, including opportunistic infections, cancer,

and neurological disorders, usually leading to death of the animal (Bendinelli *et al.*, 1995).

FIV: A Model for HIV

The FIV-infected cat is used as a small animal model for the study of pathogenesis and transmission of HIV infections (English *et al.*, 1993). FIV and HIV share similar biological, genetic, and structural characteristics. As in other lentiviruses, the FIV genome is composed of three large open reading frames (gag, pol, env), that encode internal structural proteins, viral enzymes, and envelope glycoproteins, respectively. Additional overlapping genes include vif, rev, and ORF (tat in the HIV genome), which encode accessory proteins that function in regulating or optimizing viral replication. HIV encodes three additional accessory genes not found in the FIV genome, vpr, vpu, and nef (Bendinelli *et al.*, 1995). FIV infection progresses in phases similar to those identified in HIV pathogenesis, beginning with a primary state of infection and progressing a more severe, often fatal phase. An essential difference between HIV and FIV is their use of receptors for binding the host cell. The primary receptor for HIV is CD4, whereas the primary receptor for FIV is CD134, an activation marker on T cells (Willett *et al.*, 2006). However, the viruses share the same co-receptor, CXCR4, a chemokine receptor typically found on activated T cells (Willett *et al.*, 1997b). Both viruses infect T lymphocytes and macrophages, causing syncytium formation (Brunner & Pedersen, 1989), and an immunocompromised condition that results from lymphocyte apoptosis and $CD4^+$ T cell depletion (Bendinelli *et*

al., 1995; Willett *et al.*, 1997a). FIV tropism is broader than that of HIV, infecting CD4 and CD8 lymphocytes, monocytes-macrophages, astrocytes, and microglial cells. FIV infection of CD4+, CD8+, and a variety of interleukin-2 dependent and independent T cell lines, results in apoptosis, ballooning of cells, or syncytium formation (Bendinelli *et al.,*1995). English et. al. (1993) discovered that the FIV provirus was also present in T-null cell populations. These T-null cells include natural killer cells and IL-2 activated LAK cells. FIV infection, like HIV, reduces expression of the CD4 antigen in CD4+ T cells.

Mother to Child Transmission (MTCT) and Pregnancy Outcome

Globally, 1.5 million children are infected with HIV annually, and 33.3% of these children will die from AIDS (UNAIDS/WHO, 2000). At least 91% of pediatric AIDS cases are a result of perinatal transmission occurring either *in utero*, during delivery, or lactogenically (CDC, March 2007). Mother to child transmission of HIV-1 occurs in 14 to 48% of infected, pregnant women worldwide (John & Kreiss, 1996) with a range of 13 to 32% in the United States (Dabis *et al.*, 1993). Anti-retroviral drug therapy has reduced vertically transmitted infections in developed countries by reducing maternal viral load (Kind *et al.*, 1998; Mandelbrot *et al.*, 1998); however, cost and availability limit access to anti-retroviral drugs for many women in impoverished nations (Newell, 2000).

Due to a high virus load in saliva, the major route of natural transmission of FIV is through bite wounds, and therefore, free-roaming toms are the most

frequently infected populations. Saliva contains free virus, as well as a high content of virus infected cells and free cellular debris (Bendinelli *et al.*, 1995). Seminal transmission (Lunghi *et al.*, 2007) was demonstrated by detection of FIV gag protein expression in peripheral blood leukocytes weeks after artificial insemination of queens (Jordan *et al.*, 1996). While the frequency of sexual, lactogenic, and natural transplacental transmission remains unclear, vertical transmission among experimentally-infected queens is very efficient and strain dependent (Bendinelli *et al.*, 1995). O'Neil *et al* (1996) reported 71% infection of the offspring delivered from queens who were chronically infected with either FIV-B-2542 or FIV-AB-2771, accompanied by a low CD4 cell count in 6 of the 19 FIV-infected kittens. Rogers and Hoover (2002) concluded that queens infected with FIV-C-Pgmr strain produced offspring in which 92% of the fetuses and all placentas were infected with the virus, while queens infected with a second strain, FIV-B-2542, produced offspring in which 60% of the fetuses and 96% of placentas were infected. The rate of transplacental infection increases with gestational age, with fetal infection prevalences of 0%, 5%, 38%, and 60% at 3 weeks, 5 weeks, 7 weeks, and 9 weeks gestation, respectively (Rogers & Hoover, 2002). Weaver *et al* (2005) reported 95% fetal infection at 8 weeks gestation when queens were infected with FIV-B-2542 prior to breeding, and viral infection was accompanied by a high rate of fetal non-viability. We recently reported fetal infection in 14 of 14 representative placentas and 12 of 14 accompanying fetuses by week 3-4 gestation. In this study, uninfected queens produced 41 viable and 2 non-viable fetuses, whereas the infected queens

produced 21 viable and 6 non-viable fetuses. The litter size was reduced and reproductive failure was significantly higher in FIV-infected queens when compared to control cats (Boudreaux *et al.*, 2009).

Viral Contamination of Amniotic Fluid

The amnion, a fluid-filled membrane surrounding the fetus, provides buoyancy to protect against shock, provide freedom of movement, and allow symmetrical development. Infection of amniotic fluid by various viruses has been reported. Enders et. al reported that parvoviral DNA was detected in 20% (12 of 60) of the human amniotic fluid samples from fetuses with hydrops, anemia, or isolated effusion. The viral load in the positive cases exceeded a concentration of 10^7 copies/mL (Enders *et al.*, 2009). The amniotic fluid of 92 Iranian women undergoing cesarean section was evaluated for the presence of human cytomegalovirus (HCMV) DNA sequences. HCMV was detected in 4 of 15 amniotic fluid samples (Ziyaeyan *et al.*, 2007). In another study using nested PCR to detect viral DNA, HCMV load was detected at a higher concentration in amniotic fluid than in plasma (Gouarin *et al.*, 2002). Using real-time PCR, HCMV was detected in 100% of amniotic fluid samples from the patients between 8-38 weeks of gestation whose fetuses were shown to have developmental abnormalities by ultrasonography (Gouarin *et al.*, 2002). Contamination of amniotic fluid with HIV was demonstrated in infected mothers (Mundy *et al.*, 1987). Jaspan *et. al* (2004) detected the p24 antigen in the amniotic fluid of 8 of 10 HIV-1 infected mothers at delivery. In a study by Mohala et. al (2005), HIV

contamination of amniotic fluid did not occur by 38-40 weeks of gestation in women undergoing anti-retroviral therapy. Rupture of the amniotic membrane during vaginal delivery is considered a likely mode of HIV contamination of these fluids from the cervix. Thus, delivery by cesarean section minimizes the potential for amniotic fluid contamination and reduces the risk of fetal exposure to the virus.

Immunological and Neutralizing Responses

The replication of HIV and FIV is restricted by both cell-mediated and humoral immune responses in the infected host. Cats acutely infected with FIV for as little as three to six weeks develop virus-specific antibody responses. These antibodies limit the infection both in vitro and in vivo, block cell-associated and cell-free infection, and prevent virus replication in previously-infected cats. Burkhardt et. al reported that the passage of cell-free FIV across a mucosal surface can be hindered by IgG in plasma elicited during early FIV infection (Burkhard & Hoover, 2005).

FIV epitopes were shown to stimulate neutralizing antibodies which suppress infection (Tozzini *et al.*, 1993). These antibodies were sometimes evident at two to six weeks after infection and persisted during a three year period of observation (Bendinelli *et al.*, 1995). Neutralizing antibodies in the sera of HIV-1-infected pregnant women were proven to reduce the risk of vertical transmission (Scarlatti *et al.*, 1993). Kamara *et al* (2005) reported that at a low plasma dilution (10^{-1}), maternal antibodies were capable of reducing HIV-1

infection, whereas at a higher dilutions (10^{-3} and 10^{-5}), the antibodies enhanced viral replication. Parren *et. al* (2001) discovered that HIV-1 isolates were neutralized by the sera collected from pregnant women of long-term infected patients. Barin *et al* (2006) showed that 79% of 28 serum samples had neutralizing activity against HIV-1.

Jaspan *et al* (2004) reported that the amniotic fluid of HIV-infected women contained a higher neutralizing antibody titer than plasma and was capable of reducing infection. The concentration of anti-HIV-1 antibody was similar between amniotic fluid and plasma; however the total concentration of antibodies in amniotic fluid was significantly lower than in plasma. In the SIV-infected rhesus macaque model, animals expressed significantly higher ratios of SIV p27-specific antibodies in the amniotic fluid than plasma. These studies indicate that amniotic fluid may be more important in protecting the fetus from vertical transmission, via neutralization, than is plasma. The amniotic fluid from infected humans possessed the ability to block HIV infection, but the extent of the neutralization activity could not be determined due to prior treatment of the patient with the anti-retroviral drug zidovidine. The amniotic fluid of the majority of the SIV-infected macaques, in the absence of retroviral drugs, contained neutralizing antibodies to SIV. While a higher antibody titer to HIV was found in amniotic fluid than serum of both HIV-infected pregnant women and SIV-infected rhesus macaques (Jaspan *et al.*, 2004), this type of comparison has not been conducted among FIV-infected cats.

Significance and Purpose of Study

The role that amniotic fluid play in MTCT of HIV remains unclear. There is no clear correlation between the presence of virus in amniotic fluids and fetal infection, nor does the presence of virus neutralizing antibodies in AF eliminate vertical transfer (Bal *et. al.,* 1996; Dickerson *et, al.,* 2006; Husson *et. al.*, 1995). It is difficult to address this enigma in humans because of difficulty in obtaining AF while membranes are intact, prior to their contamination by maternal blood. In addition, most infected women are treated with anti-retroviral drugs which artificially reduce virus load in these fluids. We used the FIV-infected cat model to address this question because this system is an accepted small animal model for lentivirus pathogenesis and transmission, because the virus is efficiently transmitted in utero under experimental conditions. In addition, the cats typically produce multiple offspring/litter, allowing the opportunity to obtain multiple samples from the same cat. We obtained AF and sera from early- and late-term pregnancies from queens that produced very high rates of fetal infection. AF was evaluated for viral load by measuring viral RNA and p24 antigen. AF and corresponding sera were tested for FIV-specific antibody by ELISA assays, and randomly-selected AF were examined for virus neutralizing activity using a syncytium reduction assay. Herein, we report that FIV was not detected in AF, but both AF and sera were positive for FIV-specific antbody. It is interesting that early gestation AF and one corresponding serum had VN activity, while late-term AF did not. The data suggest that the amniotic fluids do not play an important role in fetal infection.

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CHAPTER II

ANALYSIS OF AMNIOTIC FLUID AND MATERNAL SERA FROM FELINE IMMUNODEFICIENCY VIRUS (FIV)- INFECTED CATS FOR VIRUS-SPECIFIC ANTIBODY AND VIREMIA

Abstract

FIV, a lentivirus related to HIV, produces a syndrome in the infected cat that closely parallels that of the HIV-infected human. Thus, the FIV-infected cat is used as a small animal model of HIV pathogenesis. Both viruses are vertically transmissible, resulting in fetal infection and increased reproductive failure. Infected amniotic fluids (AF) are a potential source of fetal infection. Therefore, virus-neutralizing (VN) antibodies, which may be present in AF, may play a role in limiting fetal infection. We hypothesized that amniotic fluids may be contaminated with FIV and contain VN antibodies. Fetuses were delivered from FIV-infected queens by cesarean section either at week 3-4 or week 8 of gestation. AF were aseptically aspirated from intact fetal membranes and frozen. Analysis of fetal RNA or DNA for the presence of FIV revealed a very high rate of fetal infection. All AF were tested for viral p24 antigen using a commercial ELISA kit and for viral RNA using real time RT-PCR. FIV was below the level of detection by both assay methods. Randomly-selected AF from early and late

gestation were tested for anti-viral antibody using two types of commercial ELISA kits. We detected anti-FIV p24 antibody in most early- and late-term AF (63% and 61%, respectively). Corresponding sera from early-term queens were tested for anti-viral antibody using Western Blot analysis and a commercial ELISA kit. The serum samples were positive for the FIV-specific antibody by Western blot, however the commercial ELISA was less sensitive (33% positive). Randomly-selected AF and sera were further evaluated for neutralizing antibody activity by performing a syncytium-reduction assay in FIV-infected Crandell feline kidney (CrFK) cells. Medium containing serially-diluted AF or maternal sera was added to FIV-infected CrFK cells in 24 well plates; the cells were incubated one week. The cells were stained with Giemsa, and syncytia were counted. The number of syncytia was significantly decreased in three early-term AF and a parallel serum, but not the late-term AF. Interestingly, although AF contained no detectable virus but did contain VN activity, maternal-fetal infection occurred frequently. Thus, AF appears to play no appreciable role in FIV vertical transmission.

Introduction

Globally, 1.5 million children are infected annually with HIV, and 33.3% of these children will die from AIDS (UNAIDS/WHO, 2000). At least 91% of pediatric AIDS cases are a result of perinatal transmission occurring either *in utero*, during delivery, or lactogenically (CDC, March 2007). MTCT of HIV-1 occurs in 14 to 48% of infected, pregnant women worldwide (John & Kreiss,

1996) with a range of 13 to 32% in the United States (Dabis *et al.*, 1993) in the absence of anti-retroviral therapy.

The role of amniotic fluids (AF) in HIV mother-to-child (MTCT) transmission remains unresolved. HIV contamination of AF has been reported (Jaspan *et al.*, 2004; Mundy *et al.*, 1987). Virus was detected in 28% of 101 oropharyngeal or gastric aspirates from newborns from the French Perinatal Cohort Study of MTCT, revealing frequent oral exposure to the virus in utero (Mandelbrot *et al.*, 1999). On the other hand, HIV contamination of amniotic fluid did not occur by 38-40 weeks of gestation in a group of women undergoing anti-retroviral therapy, but rupture of the amniotic membrane during vaginal delivery may allow HIV contamination of these fluids from the cervix (Mohlala *et al.*, 2005). Cesarean delivery minimizes the potential for amniotic fluid contamination and reduces the risk of fetal exposure to the virus.

Numerous investigators reported HIV-1 neutralizing antibody in maternal serum or plasma (Barin *et al.*, 2006; Kamara *et al.*, 2005; Parren *et al.*, 2001). Neutralizing antibodies in the sera of HIV-1-infected pregnant women reduced the risk of vertical transmission (Scarlatti *et al.*, 1993). Likewise, FIV epitopes were shown to stimulate neutralizing antibodies which suppress infection (Tozzini *et al.*, 1993). These antibodies were sometimes evident at two to six weeks after infection and persisted during a 3 year period of observation (Bendinelli *et al.*, 1995).

HIV-1 and SIV-specific antibodies were detected in both plasma and amniotic fluids from their respective host species. However, AF obtained from

HIV-infected women could not be used in virus neutralization assays because they were collected from women who had been treated with zidovudine during pregnancy. The potential presence of the drug in AF complicates the interpretation of the role of VN antibodies in reducing viral replication. This complication limits the usefulness of AF collected from most infected women in the U.S. or other developed nations who usually receive anti-retroviral drugs during pregnancy. Using samples collected from SIV-infected rhesus macaques which had not been treated with anti-retroviral drugs, the AF of five of six animals contained neutralizing antibodies to SIV. The one animal whose AF did not contain VN activity produced an infected infant, suggesting that the VN antibodies protected the infant.

We obtained AF and sera from early- and late-term pregnancies from FIV-infected queens that produced very high rates of fetal infection. AF were evaluated for viral load by measuring viral RNA and p24 antigen. AF and corresponding sera were tested for FIV-specific antibody by ELISA assays, and randomly-selected AF were examined for virus neutralizing activity using a syncytium reduction assay. Herein, we report that FIV was not detected in AF, but both AF and sera were positive for FIV-specific antbody. It is interesting that early gestation AF and one corresponding serum had VN activity, while late-term AF did not. The data suggest that the amniotic fluids do not play an important role in fetal infection in the FIV-infected cat.

Materials and Methods

Animals and Virus

All cats used in this study were reproductively-mature, specific pathogen-free (SPF) animals obtained from a commercial cattery. Groups of ten cats were inoculated with a feline plasma pool containing FIV-B-2542 (provided by E.A. Hoover, Colorado State University); groups of ten cats were uninoculated to serve as negative controls. FIV infection in the inoculated group was confirmed using PCR and serology, then the cats were allowed to naturally breed with SPF males. Pregnancies were confirmed by ultrasonography. Pregnancies of infected and control groups were terminated at week 3-4 (early gestation study) or week 8 (late gestation study) by cesarean section.

Collection of Amniotic Fluid and Serum

The amniotic fluid within each fetal membrane was collected by puncturing the membrane with a 21 g needle and aspirating into a syringe. Caution was taken to prevent infected maternal blood from contaminating the amniotic fluid. Fetuses, placentas, amniotic fluids, and maternal blood (serum, plasma, leukocyte DNA and RNA), collected at each delivery, were frozen at -80ºC until needed. Whole blood was drawn at biweekly to monthly intervals until delivery for the collection of serum, plasma, and peripheral blood lymphocytes.

FIV Antibody Detection using SNAP FIV/FeLV ELISA

Infected amniotic fluid samples were selected randomly for analysis using the SNAP FIV/FELV ELISA kits (IDEXX Laboratories; Westbrook, ME) to detect FIV antibody in amniotic fluid. Briefly, three drops of amniotic fluid were added to a solution containing anti-feline IgG conjugated to horseradish peroxidase, and the mixture was added to the SNAP device. The device was "snapped" to allow interaction of captured antibody complex with the enzyme substrate. AF that contained FIV-specific antibody produced a blue dot in the appropriate position on the membrane.

FIV Antibody Detection using the Petchek FIV Antibody Test Kit

FIV antibody in all amniotic fluid and the corresponding serum samples were determined using the PetChek FIV Antibody Test Kit (Idexx Laboratories; Westbrook, ME). Briefly, this sandwich ELISA assay utilized a 96-well microtiter plate, coated with FIV antigen. Samples from FIV-infected cats were added to the well, along with the appropriate positive and negative controls included in the kit. The samples were incubated with the horseradish peroxidase (HRPO) labeled FIV antigen. The preparation was incubated and washed to remove unbound antigen, then the enzyme substrate/chromagen solution was added. Color development was detected using an ELISA plate reader at a wavelength of 650 nm. Positive reactions were subjected to a confirmatory procedure. Color development was proportional to the amount of FIV in the sample. Sample to

positive ratios (S/P) were determined according to kit instructions. A threshold value (0.5) was set, and all S/P ratios above 0.5 were considered positive.

Whole Virus Purification

Supernatant and cell lysate collected from FIV-infected T cell cultures (MCH 5-4, kindly provided by Dr. John Elder) were centrifuged at 11,750 x g in a GSA centrifuge at 4°C for 20 min to remove cellular debris. The clarified supernatant was centrifuged at 23,000 x g for 90 min at 4°C using an SW27 rotor and a Beckman L8-80M Ultracentrifuge. The supernatant was discarded, and the tubes were inverted and allowed to drain. Pellets containing virus were resuspended in 200 ml of PBS and stored at -80°C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The 12% polyacrylamide gels used for the separation of proteins were composed of 12% acrylamide/bis, 1.5 M Tris-HCl (pH 8.8), 0.5 M Tris-HCl (pH 6.8), 10% SDS, 10% ammonium persulfate, and TEMED. The stock virus was diluted 1:4 in sample reducing buffer (0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% 2-mercaptoethanol, 1% bromophenol blue). Electrophoresis was done using the Mini Protein II system (Bio-Rad Laboratories, Hercules, CA), according to standard procedure (Laemmli, 1970). Electrophoresis was conducted at 100 volts for ~1 h, followed by transfer of proteins to nitrocellulose membranes (Micron Separations Inc., Westborough, MA). We performed the transfer using the Towbin buffer (25mM Tris, 192 mM glycine, 20% methanol, pH 8.3) and a semi-dry transfer apparatus (BioRad Laboratories; Hercules, CA) at 15 volts for

0.5h. Proteins on the membrane were visualized by soaking the membrane for 5 min in Ponceau S, followed by destaining in multiple changes of distilled water. The nitrocellulose was vertically cut into strips for use in Western blotting.

Western Blot Procedure

The nitrocellulose strips containing immobilized protein were incubated for at least 2 h in 5% Blotto (5 g Carnation non-fat dry milk in Tris-saline tween buffer) (TS-T; 10 mM Tris, 15 mM NaCl, 0.05%, pH 8.6) to prevent nonspecific binding of antibody to the nitrocellulose. The sample sera, along with a positive and negative control, was added to each well and allowed to incubate overnight. After removing the blotto/serum mixture and washing the strips, the secondary antibody, goat anti-cat IgG peroxidase (ICN Pharmaceuticals, Aurora, Ohio) was diluted (1:1000) and added to each well, and the strips were incubated 1-2 h. After washing the strips, an enzyme substrate/chromagen solution (0.02 g 4-chloronaphthol dissolved in 5 ml of methanol, 0.05 M Tris pH 6.8, and 12 µl H_2O_2) was added. The reaction is stopped by rinsing with distilled water.

CrFK Cell Culture and Infection

Crandell Feline Kidney cells (CrFK) were cultured in complete CrFK medium containing Minimum Essential Medium (Gibco), 1.5 g/L sodium bicarbonate, 1.0 mM sodium pyruvate, 1.0 mM non-essential amino acids, 10% antibiotics, and 10% fetal calf serum. Cells were infected with FIV-B-2542 using infectious pooled plasma collected from previously-infected cats. The inoculum,

diluted 1:10 in medium, was added to the cell monolayer and incubated for 1 h at 37°C. The inoculum was removed and replaced with complete culture medium.

Virus Neutralization Assay (Syncytium Reduction Assay)

The VN was done according to a modification of the procedure of Jaspan et al (2004). Six samples of amniotic fluid and the corresponding serum samples, positive for FIV antibodies, were heat inactivated at 56ºC for 30 min. Infected Crandell Feline Kidney (CrFK) cells were added to 12 wells and non-infected CrFK cells were added to 8 wells (density 5 x 10^4 cells/well) of a multi-well plate, according to the following diagram (Figure 2.1). Amniotic fluid samples were diluted in a four-fold series, mixed with the cells, and incubated in a 5% CO_2 incubator at 37ºC for 7 days. Following incubation, these reaction mixtures were removed; the cells were fixed in methanol and stained with 5% Giemsa. Wells were microscopically observed at 40X magnification for the development of syncytia.

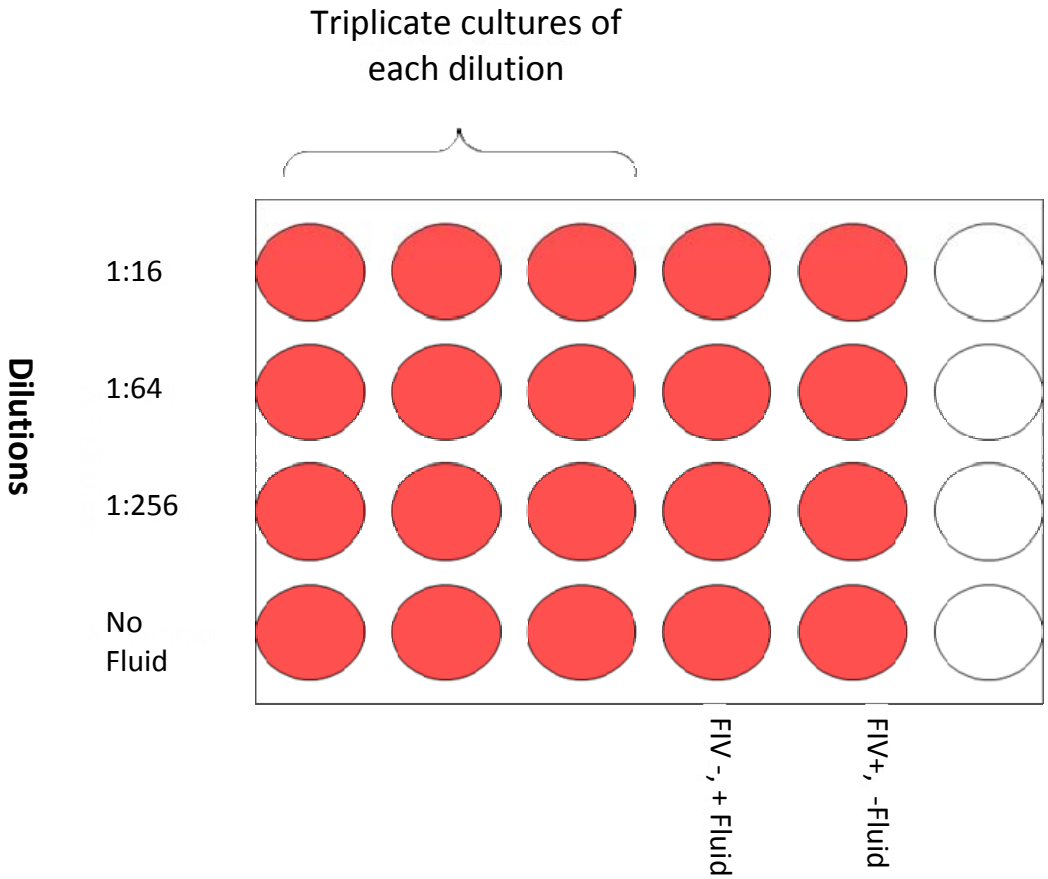

Figure 2.1 Virus Neutralization Assay Design

Quantification of FIV Antigen in Amniotic Fluid

FIV load in amniotic fluid and serum samples was determined using the PetChek FIV Antigen Test Kit (Idexx Laboratories). Briefly, this sandwich ELISA assay used a 96-well microtiter plate, coated with monoclonal antibody specific for FIV p24. Samples from FIV-infected cats were added to the wells, along with a serum and amniotic fluid sample from a control cat. Appropriate positive and negative controls, included in the kit, were used as well. After the incubation and washes, the second monoclonal antibody to p24, labeled with horseradish peroxidase, was added. The reaction was incubated and washed to remove unbound antibody, then the enzyme substrate/chromagen solution was added. Color development was detected using an ELISA plate reader at a wavelength of 650 nm. Positive reactions were subjected to a confirmatory procedure. Color development was proportional to the amount of FIV in the sample.

RNA Extraction

RNA was extracted from the amniotic fluid using the QIAamp Viral RNA Mini Kit (Qiagen). RNA concentration was measured on NanoDrop 1000 (Thermo Scientific, Waltham, MA) and stored in -80ºC until needed.

Quantification of FIV RNA using Real-Time Reverse Transcriptase (RT) PCR

Real time TaqMan primers and probes targeting the FIV *gag* gene and β-actin (housekeeping gene) were obtained commercially (MWG Biotech, Inc., High Point, NC). The primer and probe sequences and 5' and 3' probe labels are shown in Table 2.1. The 25 µL PCR mixtures contained 6 µL standard or sample,

12.5 µL of qRT-PCR Thermoscript reaction mix (Invitrogen), 0.5 µL Thermoscript Taq (Invitrogen), 1 µL β-actin forward and reverse primers (10 pmole/ µL), 1 µL of FIV gag forward and reverse primers (10 pmole/µL), and 1 µL β-actin and FIV gag probe (1 pmole/µL). Serial, 2-fold dilutions of viral RNA (1:2 to 1:64) were used to generate a standard curve. Samples and standards were analyzed in triplicate on a 96 well plate using an iCycler (Bio-Rad). The following thermal cycling parameters were used: 3 min at 95ºC, 40 cycles at 95ºC for 30 sec, and 30 sec at 60ºC.

Table 2.1 Oligonucleotides used for real-time RT-PCR gene expression analysis

Targeted genes and Gen Bank accession number	Sequence (5' → 3')	Purpose	5' Label
Gag	5'-GTATGATCGTACTCATCCTCCTGAT-3' 5'-TCTACATTGCATTCTGGCTGGT-3' 5'-AGACCACTGCCCTACTTCACTGCCG-3'	Forward Reverse Probe	____ ____ FAM
B-actin AB051104	5'-GACTACCTCATGAAGATCCTCACG-3' 5'-TCTCCTTGATGTCACGCACAATT-3' 5'-ACAGTTTCACCACCACCGCCGAGC-3'	Forward Reverse Probe	____ ____ HEX

Target probe labels: 5' FAM (6-carboxyfluorescein), 3' TAMRA (6-carboxytetramethylrhodamine), 5' HEX (hexachloro-6-carboxyfluorescein)

Statistical Analysis

Statistical analysis of FIV load and antibody levels in amniotic fluid was completed using single-factor ANOVA at the 95% confidence level.

Results

I. Viral Load and FIV-specific Antibody in Amniotic Fluids (AF)

Viral Load and FIV-specific Antibody Detection in Early-term AF Samples

Early-term amniotic fluid samples were tested for FIV load and FIV-specific antibody (Table 3.1). The majority of the samples were tested for FIV p24 antigen using the Petchek Ag Test kit. All samples were negative, indicating that virus was either absent or below the level of detection in AF. One sample from each queen was tested for viral RNA using real time RT-PCR. No viral RNA was detected in any of the samples, confirming the results of the p24 antigen assay. Using SNAP ELISA kits to detect FIV-specific IgG in nine randomly-selected AF samples, six samples were strongly positive (5111B, 0866A, 0866C, 0866E, 1893C, and 1126D) one was weakly positive (6062A), and two were negative (8035B and 6062B) (Figure 3.1).

Viral Load and FIV-specific Antibody in Late-term AF Samples

Late-term amniotic fluid samples were tested for FIV load and FIV-specific antibody (Table 3.2). All samples were tested for FIV p24 antigen using the Petchek Ag Test kit. All samples were negative, indicating that virus was either absent or below the level of detection in AF. One sample from each queen was

tested for viral RNA using real time RT-PCR. No viral RNA was detected in any of the samples, confirming the results of the p24 antigen assay. Using SNAP ELISA kits to detect FIV-specific IgG in eight randomly-selected AF samples, two samples were strongly positive (9745A and 9806A), four samples were weakly positive (9674A, 9810A, 13226E, and 9809A), and two samples were negative (9730A and 13226B).

Table 3.1 Viral Load and IgG Detection of the Early Term Amniotic Fluid Samples

Infected Cat #	Real Time PCR	Ag ELISA	FIV-specific IgG	Fetal Infection
O326	negative	negative	ND	weakly +
8035 A	ND	negative	ND	negative
8035 B (Arr)	ND	negative	negative	negative
8035 C (Arr)	ND	negative	ND	positive
8035 D (Res)	negative	negative	ND	ND
5111 A	negative	negative	ND	weakly +
5111 B*	ND	negative	strong +	weakly +
0866 A	ND	negative	strong +	positive
0866 C	ND	negative	strong +	ND
0866 E	negative	negative	strong +	ND
0866 F	ND	negative	ND	ND
1893 A	negative	negative	ND	weakly +
1893 C	ND	negative	strong +	ND
6062 A	ND	negative	very weak +	weakly +
6062 B	negative	negative	negative	weakly +
6062 C	ND	negative	ND	ND
1126 D	negative	N/A	strong +	ND

Note: (*) blood contamination; (ND) not done; (Arr) amniotic fluid from arrested fetus; (Res) amniotic fluid from arrested fetus

Table 3.2 Viral Load and IgG Detection of the Late Term AF Samples

Infected Cat #	Real Time PCR	Ag ELISA	FIV-specific IgG	Fetal Infection
9674 A	negative	negative	weak +	positive
9813 A	negative	negative	ND	positive
9810 A	negative	negative	weak +	positive
9730 A	ND	negative	negative	positive
9730 B	ND	negative	ND	positive
9730 C Res	negative	negative	ND	positive
9730 D Res	ND	negative	ND	positive
13226 A Arr	ND	negative	ND	positive
13226 B Arr	ND	negative	negative	positive
13226 C Arr	ND	negative	ND	positive
13226 D Arr	negative	negative	ND	positive
13226 E Arr	ND	negative	weak +	positive
9745 A	negative	negative	strong +	negative
9745 B Res	ND	negative	ND	positive
9745 C Res	ND	negative	ND	positive
9745 D	ND	negative	ND	positive
9745 E Res	ND	negative	ND	positive
9745 F	ND	negative	ND	positive
9806 A	negative	negative	strong +	positive
9806 B	ND	negative	ND	positive
9809 A	negative	negative	weak +	positive

(Arr) amniotic fluid from arrested fetus; (Res) amniotic fluid from arrested fetus

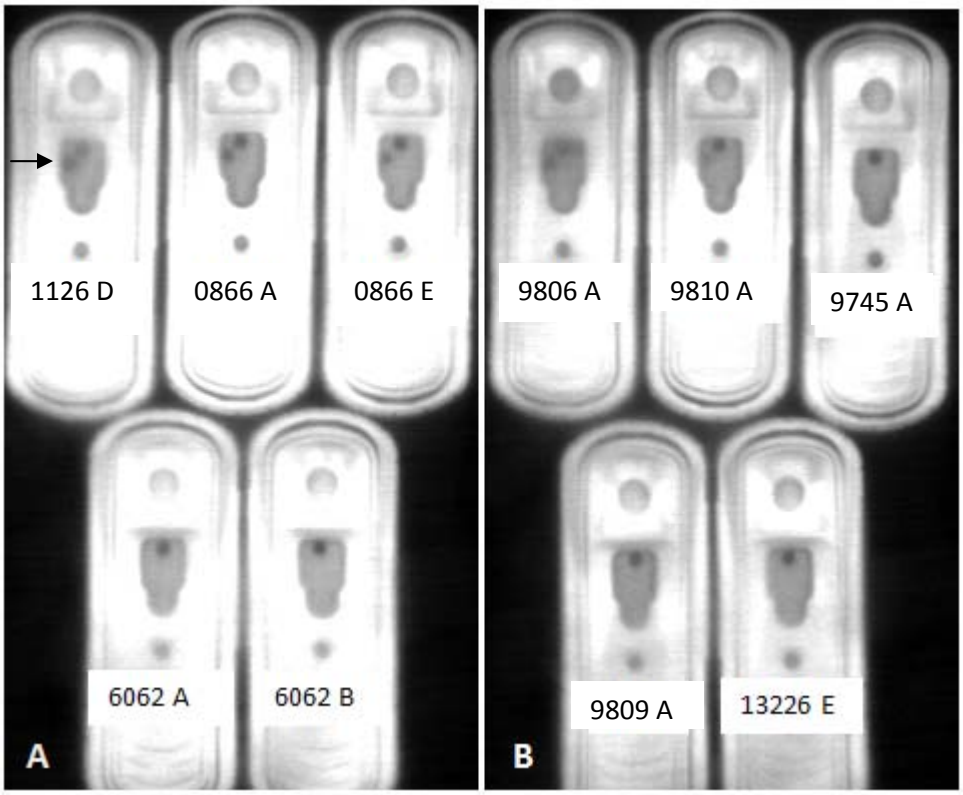

Figure 3.1 SNAP ELISAs for Early and Late Term AF Sample

Note: 8035 B, 5111B, 0866 E, 1893C, 9674A, 9730A, and 13226 B are not shown.

II. Relative Level of FIV-specific Antibody in AF

Relative FIV-specific Antibody Levels for Early-term AF

The sample-to-positive (S/P) ratios of seroreactivity to FIV p24 for all early-term AF samples, determined using the FIV Petchek Antibody test kit, are shown in Figure 3.2. Samples with an S/P ratio more than the 0.5 threshold were considered positive. Ten of the sixteen samples (63%) had a ratio higher than 0.5, and thus, were positive for FIV-specific antibodies.

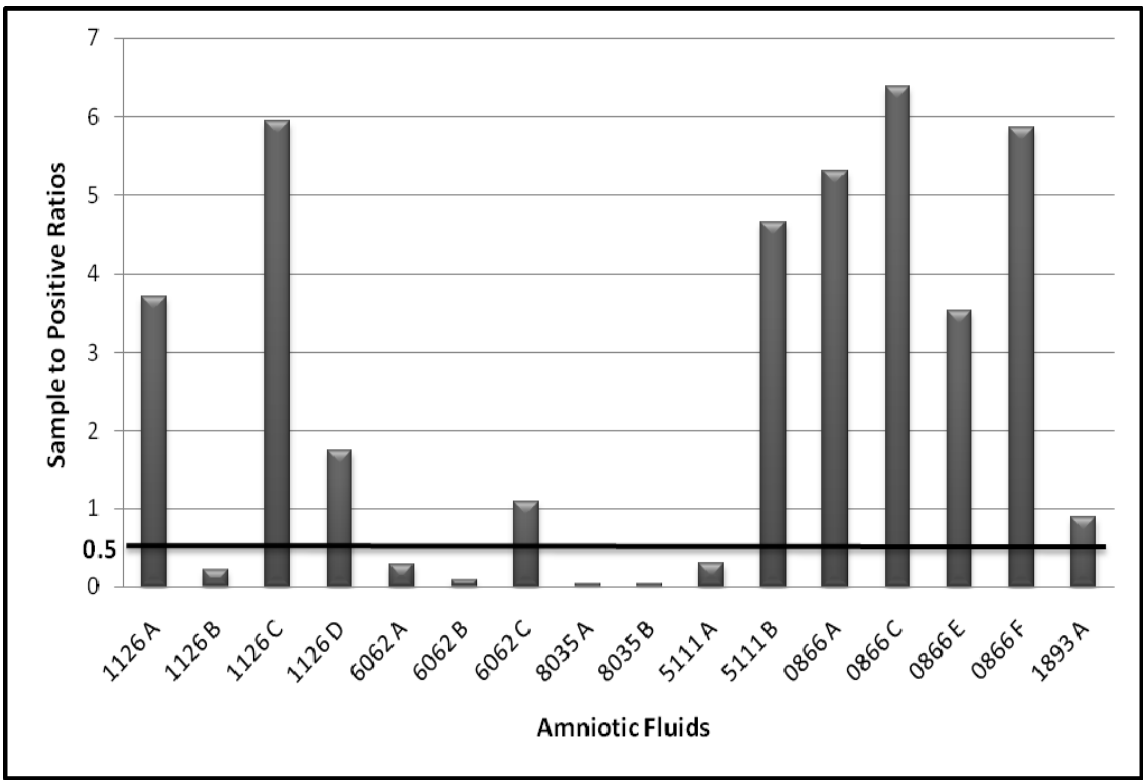

Figure 3.2: Relative Levels of FIV-specific Antibody in Early-term AF Samples. OD of 650 nm were determined for each sample and controls (included in kit), and sample to positive (S/P) ratios were calculated. The 0.5 S/P indicates the positive/negative threshold for antibody detection. All S/P ratios above 0.5 were considered positive.

Relative FIV-specific Antibody Levels for Late-term AF

The sample-to-positive (S/P) ratios of seroreactivity to FIV p24 for all early-term AF samples, determined using the FIV Petchek Antibody test kit, are shown in Figure 3.3. Samples with a S/P ratio over the 0.5 threshold were considered positive. Eleven of the eighteen samples (61%) had a ratio higher than 0.5, and thus, were positive for FIV-specific antibodies. Two of the six FIV-infected queens produced FIV seropositive AF for all fetal membranes. Only one AF sample from queen 13226 was FIV seropositive out of the five tested. These results reveal serological variability even within single queens.

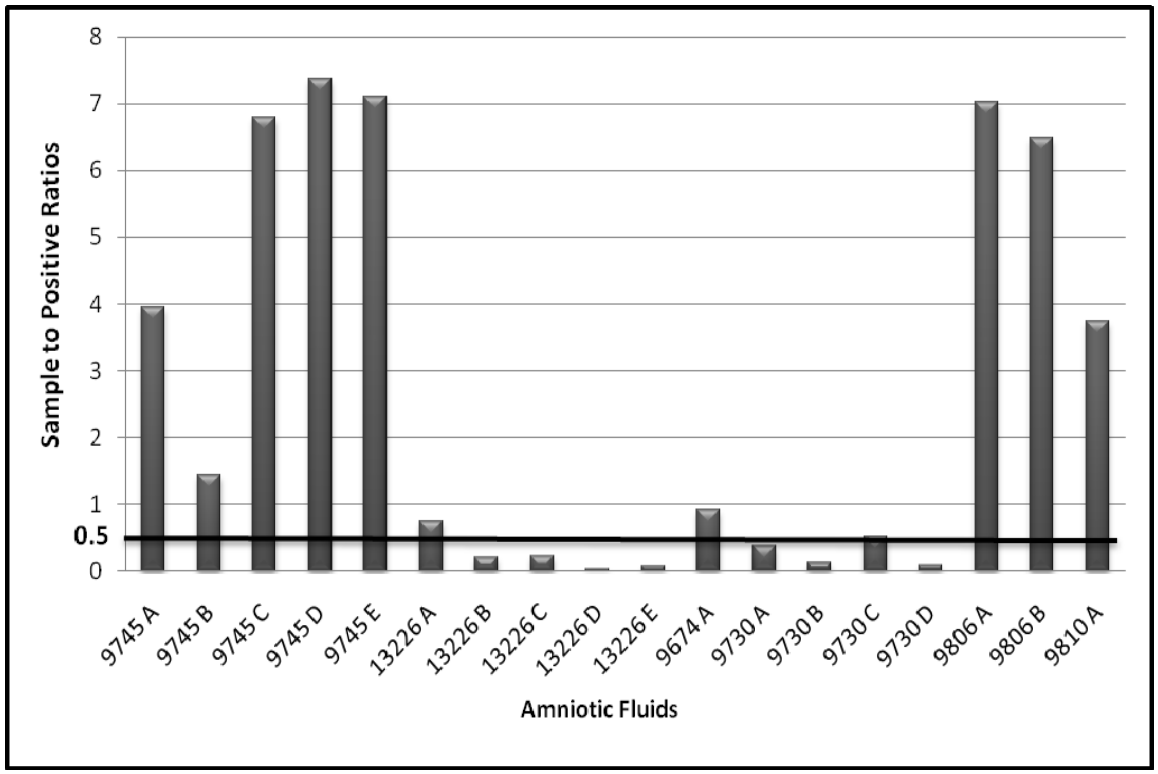

Figure 3.3: Relative Levels of FIV-specific Antibody in Late –term AF Samples. OD 650 nm were determined for each sample and controls (included in kit), and sample to positive (S/P) ratios were calculated. The 0.5 S/P indicates the positive/negative threshold for antibody detection. All S/P ratios above 0.5 were considered positive.

Comparisons of FIV Seroreactivity in Serum from Early Infection vs. Termination

The results of ELISA to detect FIV-specific antibody in serum collected from infected queens during early infection (week 8) and at termination are shown in Figure 3.4. Only two animals were seropositive using this assay (8035 and 5111), both of whom had higher antibody levels at week 8 p.i. The western blot analysis of longitudinal serum samples identified seroreactivity to FIV p24, the major immunodominant viral protein in all animals. All animals were strongly seroreactive by termination at 21-25 weeks p.i., and four of six animals were strongly positive by the week 8 p.i. bleed. Only animals 1893 and 1826 produced a weak serological response at week 8 p.i.

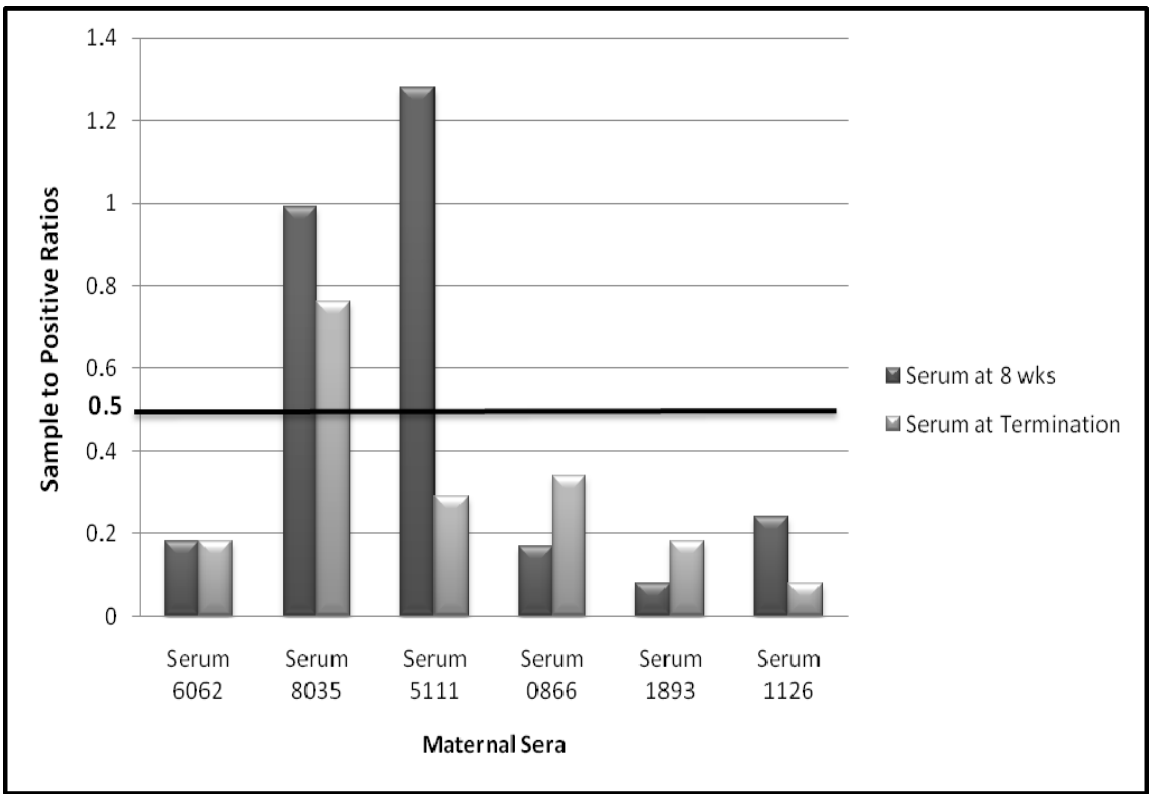

Figure 3.4: Relative Levels of FIV-specific Antibody in Serum Samples. OD 650 nm were determined for each sample and controls (included in kit), and sample to positive (S/P) ratios were calculated. The 0.5 S/P indicates the positive/negative threshold for antibody detection. All S/P ratios above 0.5 were considered positive.

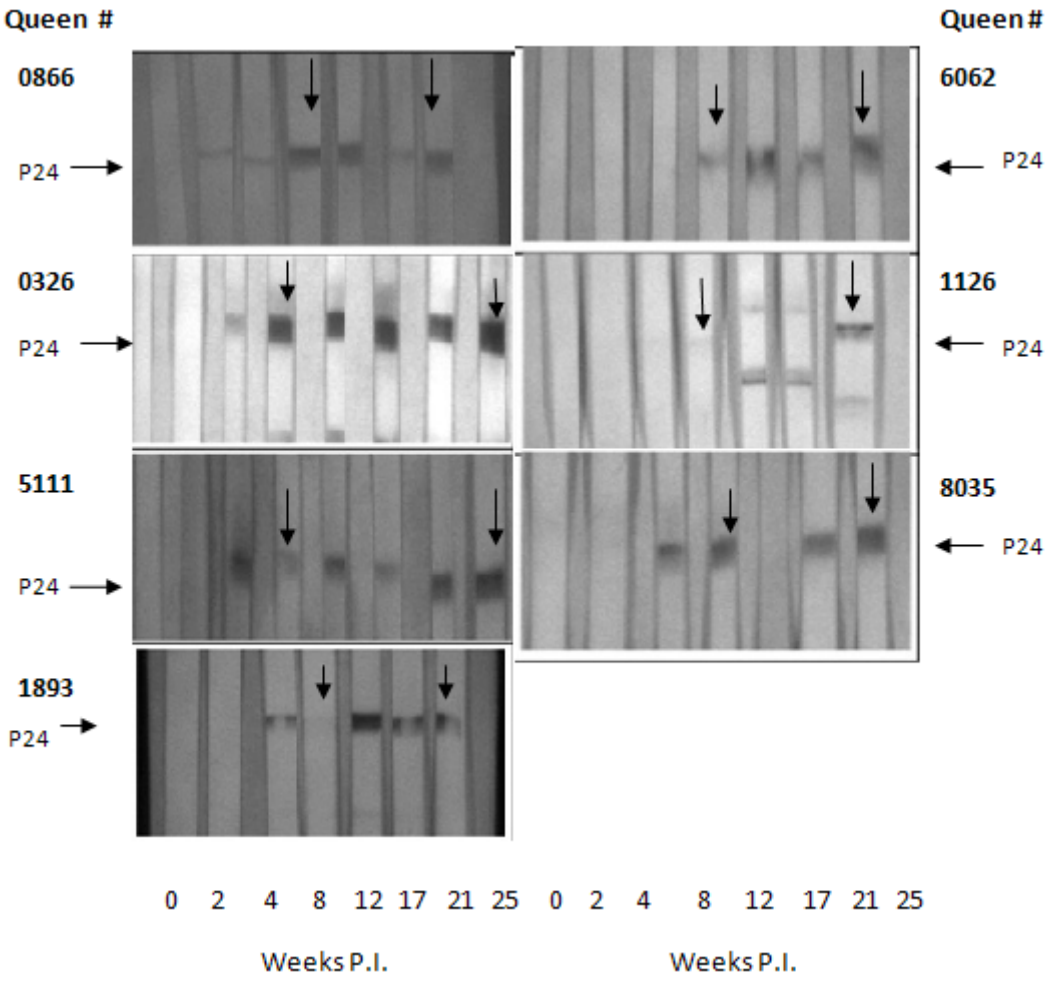

Figure 3.5: Western blot analysis of longitudinal serum samples collected from FIV-infected queens. Reactivity to the immunodominant p24 is indicated. Arrows identify strips tested with sera collected at week 8 p.i. or at the terminal bleed.

III. Syncytium Reduction with AF and Terminal Serum Samples

Early Pregnancies

Figure 3.6 is representative of the cytopathic effect that was observed with the syncytium reduction assay. In the presence of early-(A) or late-term (C) AF, syncytia were reduced or not evident in FIV-infected CrFK cells. However, in the absence of treatment with AF (B and D), syncytia were prominent in infected CrFK cells.

The results of the syncytium reduction assay for queen 0866 AF and terminal serum sample are shown in Figure 3.7. The mean syncytium counts for triplicate cultures of FIV-infected CrFK cells treated with AF or serum dilutions are graphed. For the AF (Figure 3.7A), the mean number of syncytia was significantly higher ($p=0.04$) at the 1:64 dilution of AF than the 1:16 dilution. Likewise, a significantly higher number of syncytia developed in cultures that were untreated with AF than those treated with the lowest dilution ($p= 0.03$). The data show that AF reduced viral replication and indicate that VN antibodies were present in AF from this animal. For the serum assay (Figure 3.7 B), mean syncytia were reduced with increased dilution from 1:16 to 1:256 ($p=0.02$) and 1:64 to 1:256 ($p=0.02$), while the 1:16 dilution yielded a very similar number of mean syncytia as the untreated cells. These data are opposite the expected results and are difficult to interpret.

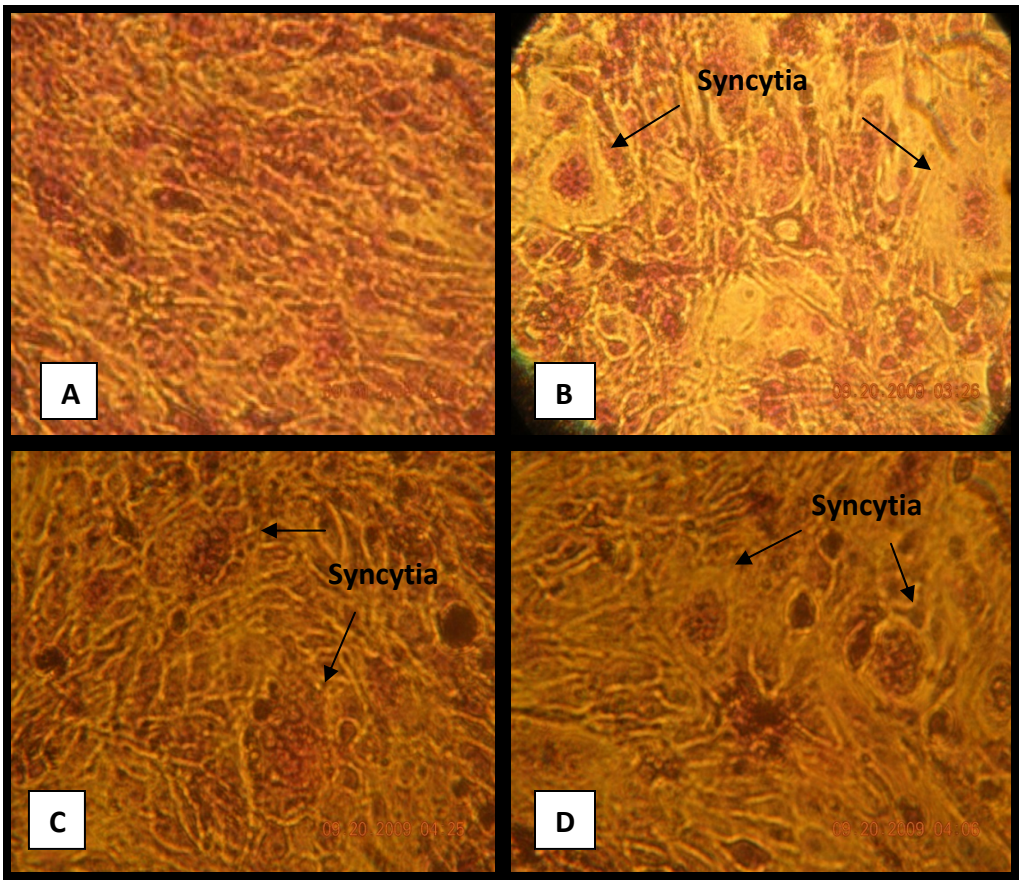

Figure 3.6: CrFK cells from Virus Neutralization Assays. Infected cells were incubated in a high dilution of early term AF (A), high dilution of late term AF (C), and cells were untreated with the cooresponding fluids (B and D).

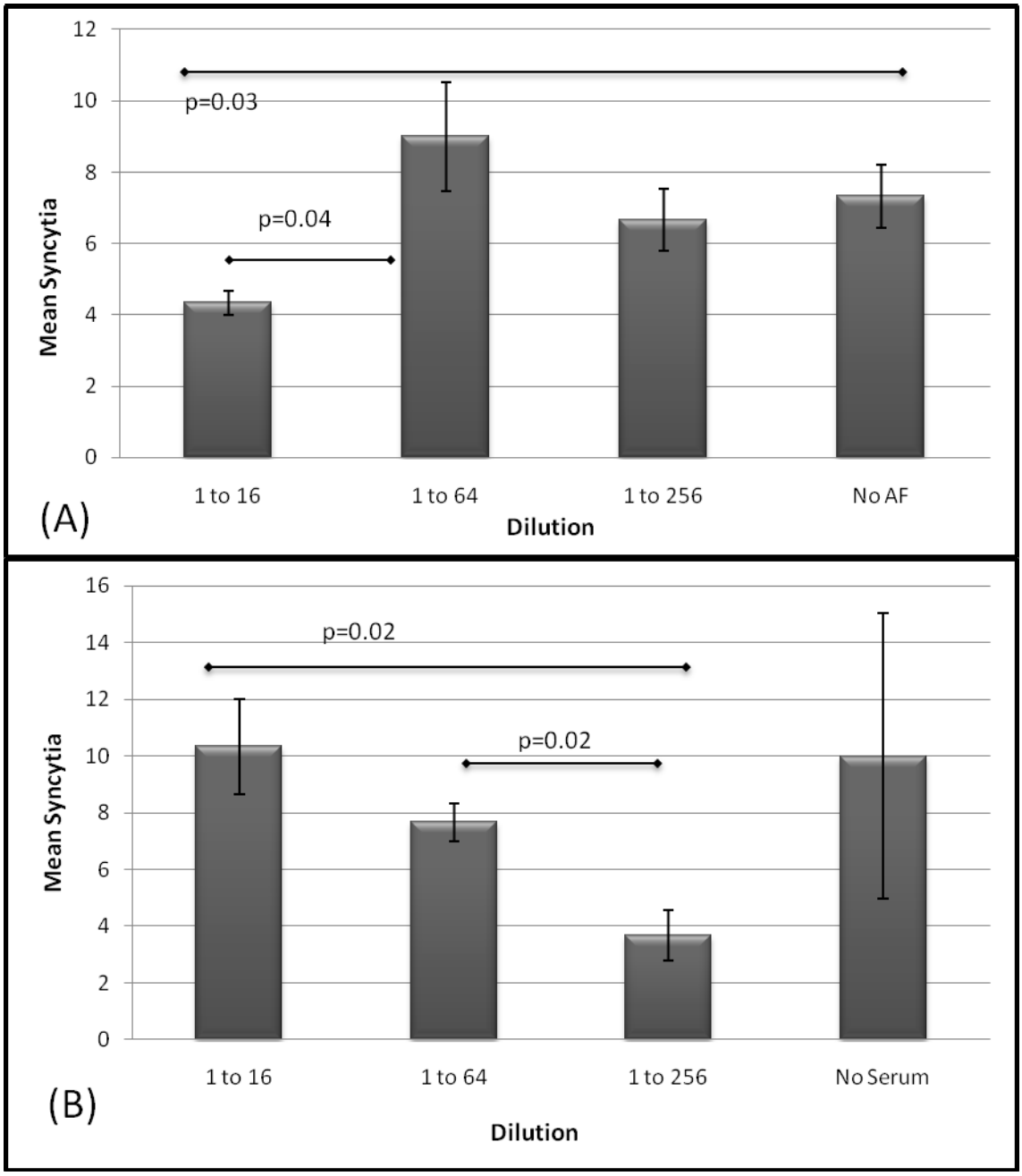

Figure 3.7: Syncytium reduction assays using AF or serum from cat 0866. The mean number of syncytia produced in FIV-infected CrFK cells treated with diluted AF (A) or serum (B) is shown as bars bracketed by the standard error of the mean. $P \leq 0.05$ were significant.

The results of the syncytium reduction assay for queen 6062 AF and terminal serum sample are shown in Figure 3.8. In comparison to all dilutions, the mean number of syncytia was significantly higher in cultures untreated with AF (Figure 3.8 A), with P values of 0.001, 0.008, and 0.03 for AF dilutions of 1:16, 1:64, and 1:256, respectively. None of the cultures treated with AF were different from each other ($p > 0.05$). Serum from this animal produced very similar results with untreated cultures yielding significantly higher numbers of syncytia than those treated with serum diluted 1:16 ($p = 0.02$), 1:64 ($p = 0.01$), and 1:256 ($p = 0.02$) (Figure 3.8 B). This animal produced VN antibody that was present in both AF and serum.

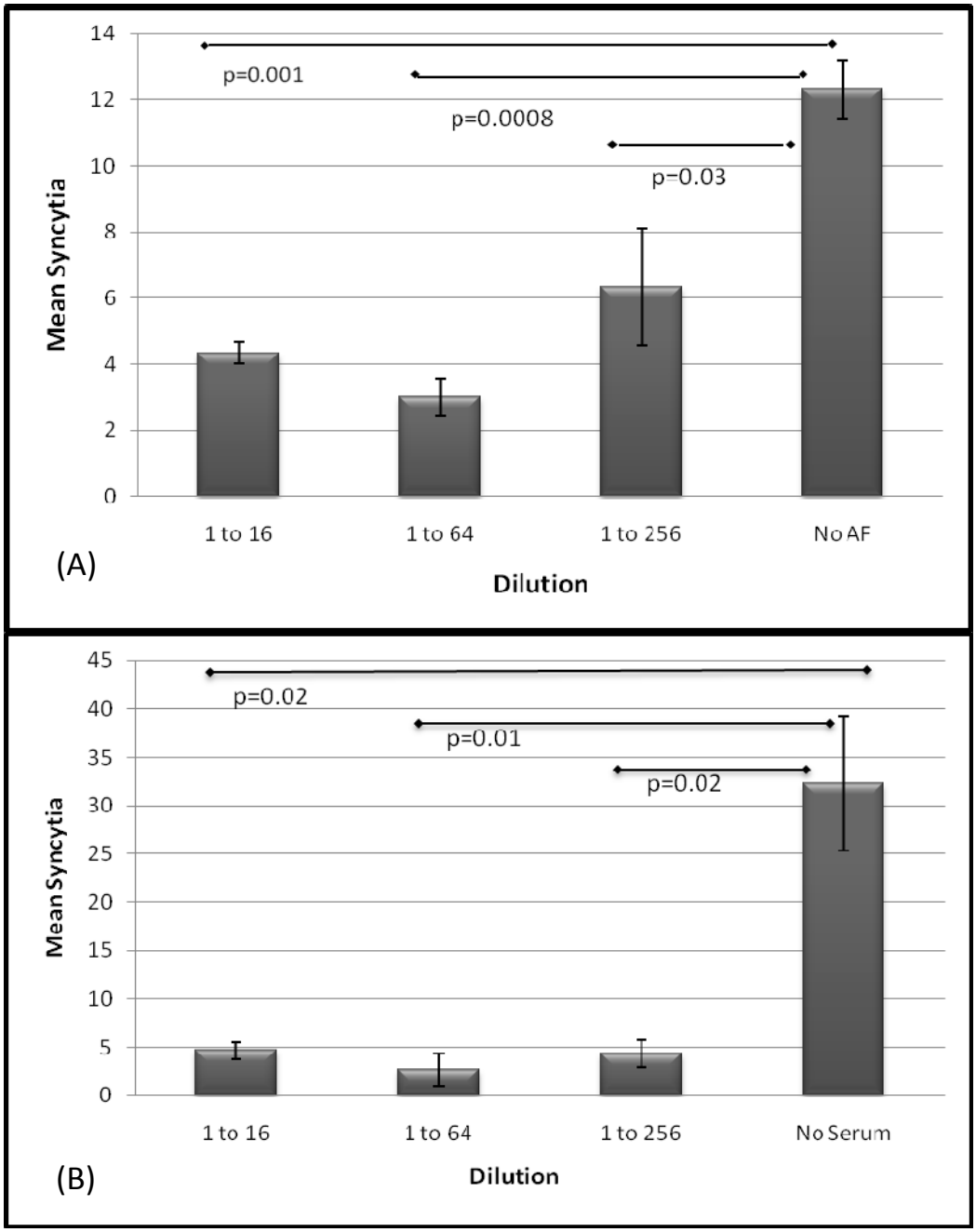

Figure 3.8: Syncytium reduction assays using AF or serum from cat 6062. The mean number of syncytia produced in FIV-infected CrFK cells treated with diluted AF (A) or serum (B) is shown as bars bracketed by the standard error of the mean. $P \leq 0.05$ were significant.

The results of the syncytium reduction assay for queen 1126 AF and terminal serum sample are shown in Figure 3.9. A dose-dependent response in syncytium reduction was seen. With the exception of the comparison of the 1:16 and 1:64 dilutions, a significant increase in syncytium occurred with decreasing levels of AF (Figure 3.9 A) . The greatest difference occurred between the highest concentration of AF (1:16) and lowest concentration of AF (no AF) ($p= 0.01$). There were no significant differences in mean syncytia between any of the serum dilutions (Figure 3.9 B). The data reveal VN capability of AF, but not serum, from this cat.

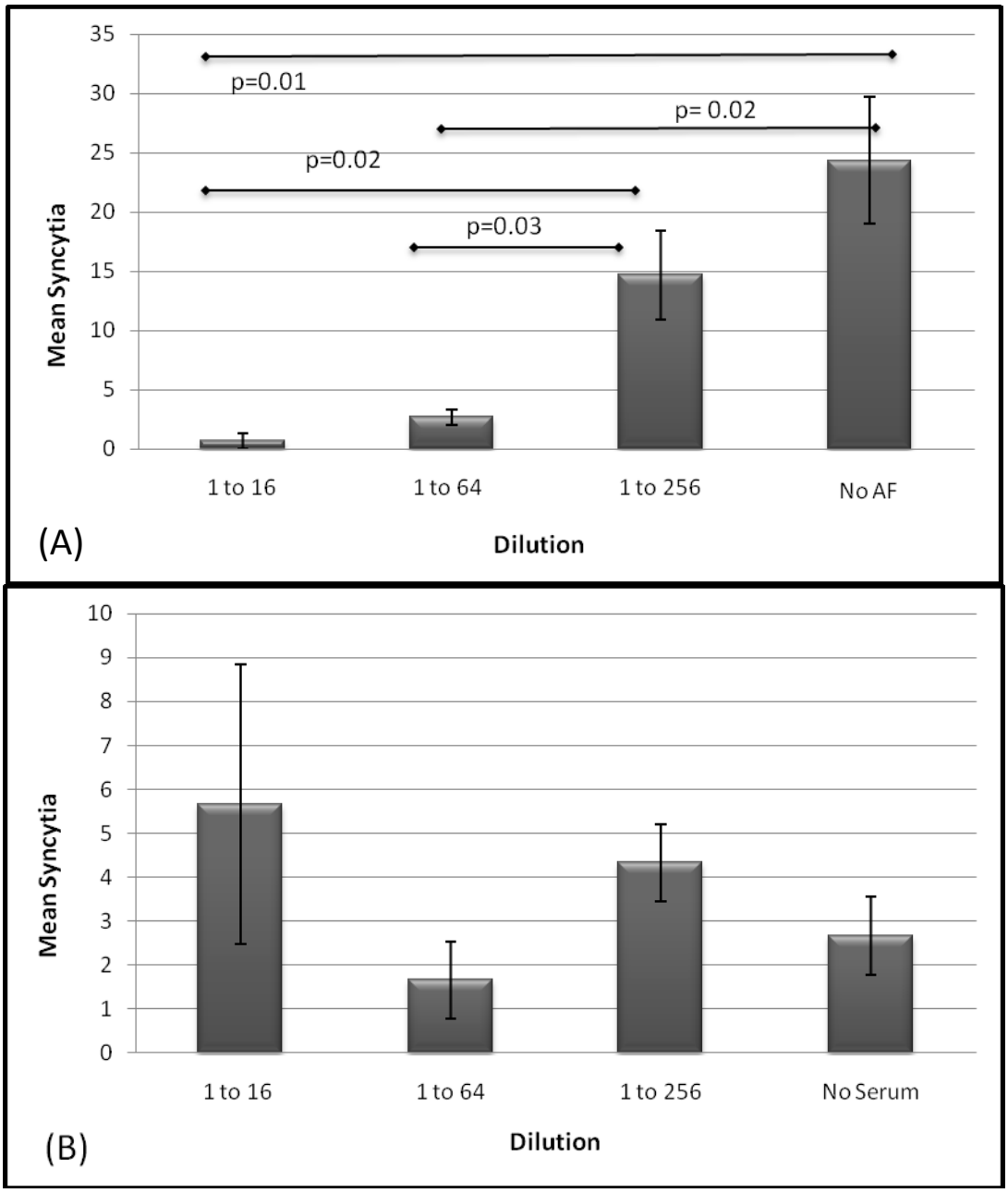

Figure 3.9: Syncytium reduction assays using AF or serum from cat 1126. The mean number of syncytia produced in FIV-infected CrFK cells treated with diluted AF (A) or serum (B) is shown as bars bracketed by the standard error of the mean. $P \leq 0.05$ were significant.

Late Pregnancies

The results of the syncytium reduction assay for queen 9674A AF are shown in Figure 3.10. Although the mean number of syncytia appeared to increase with increasing dilution, the differences did not reach the level of significance ($p > 0.05$).

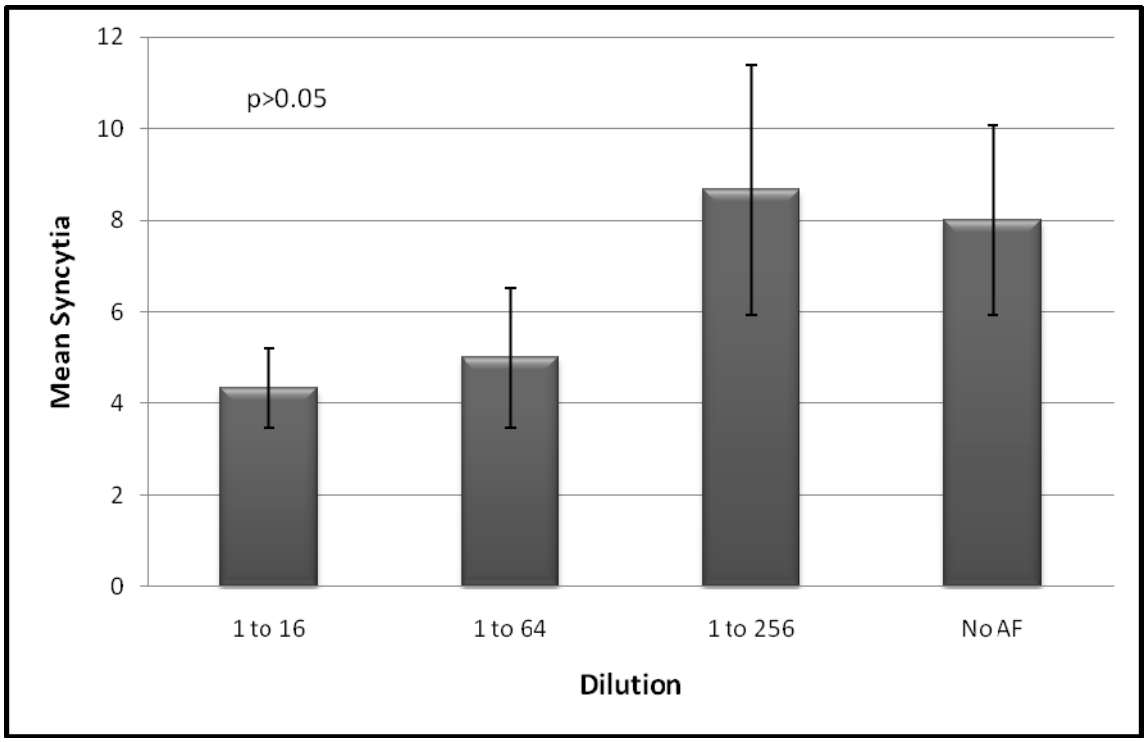

Figure 3.10: Syncytium reduction assays using AF from cat 9674A. The mean number of syncytia produced in FIV-infected CrFK cells treated with diluted AF is shown as bars bracketed by the standard error of the mean. $P \leq 0.05$ were significant.

The results of the syncytium reduction assay for queen 9810A AF are shown in Figure 3.11. A clear pattern of syncytium reduction was not evident because untreated, infected cells produced a similar number of syncytia as those treated with the highest concentration of AF. Statistical differences were not detected between any of the comparisons ($p>0.05$).

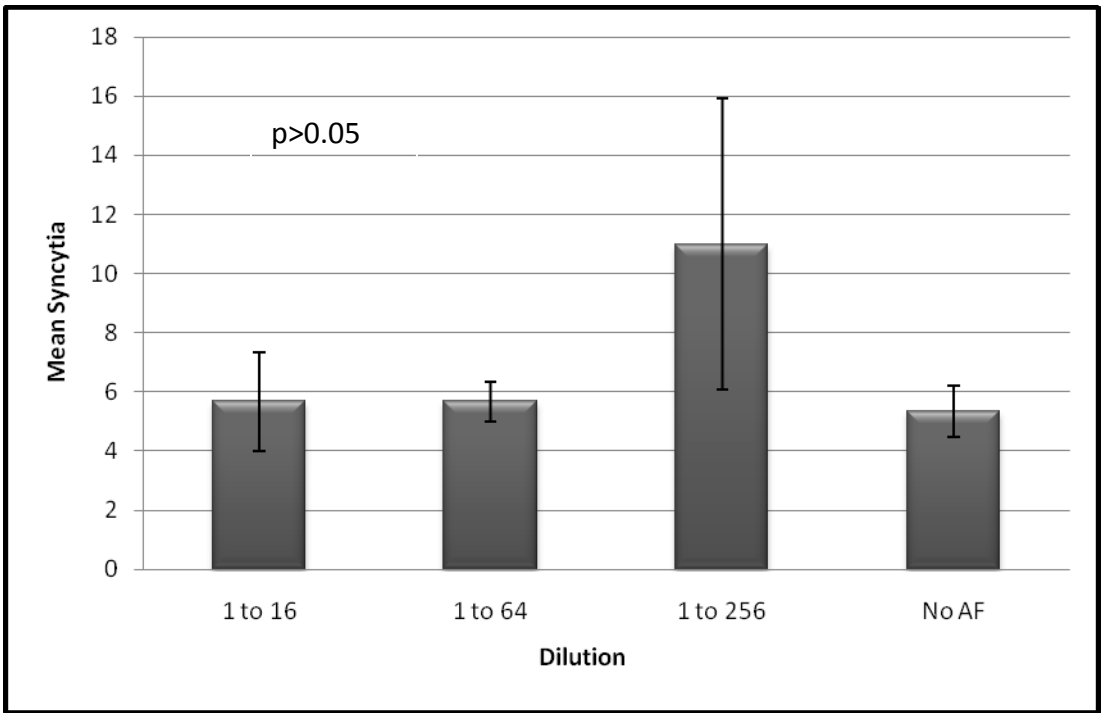

Figure 3.11: Syncytium reduction assays using AF from cat 9810A. The mean number of syncytia produced in FIV-infected CrFK cells treated with diluted AF is shown as bars bracketed by the standard error of the mean. $P \leq 0.05$ were significant.

The results of the syncytium reduction assay for queen 9806B AF are shown in Figure 3.12. Although the 1:16 dilution produced the lowest mean syncytium count, this value was not significantly different from the other dilutions due to wide variability in triplicate cultures at all dilutions.

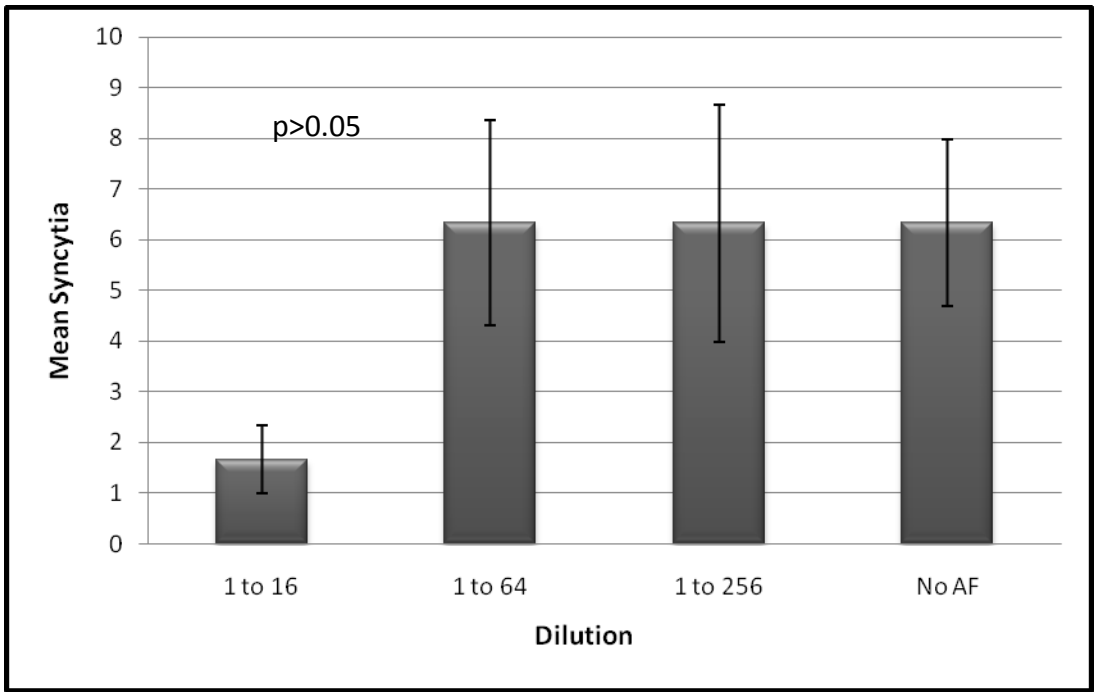

Figure 3.12: Syncytium reduction assays using AF from cat 9806B. The mean number of syncytia produced in FIV-infected CrFK cells treated with diluted AF is shown as bars bracketed by the standard error of the mean. $P \leq 0.05$ were significant.

Discussion

Fetal exposure to HIV in contaminated amniotic fluids is probably a contributor to the high rate of MTCT infection (14-48%) that occurs globally in the absence of anti-retroviral therapy (John & Kreiss, 1996). The detection of HIV in gastric aspirates of neonates indicates oral exposure of the fetus to the virus through ingestion of contaminated AF (Mandelbrot *et al.*, 1999). The potential importance of AF as a source of antenatal exposure to HIV is difficult to study in humans due to limitations in specimen collection and because HIV-infected women in developing nations are typically treated with anti-retroviral drugs, which reduce viral load. While the SIV-infected macaque provides an animal model, the data are limited with this model system due to small numbers of offspring. The FIV-infected cat model provides a good alternative animal model system due to the high rate of MTCT that can be achieved under experimental conditions and the multiple offspring produced by a single queen, which naturally expands the number of AF samples that can be collected. While this alternative animal model has been used by us and others to evaluate aspects of lentiviral vertical transmission, the role of the AF in MTCT in this system has not been extensively studied.

The purpose of this study was to examine AF samples collected at early and late stages of gestation to determine whether or not for these fluids play a role in MTCT. We evaluated the AF for the presence of FIV antigen to determine viral load, compared FIV-specific antibody reactivity, and measured virus neutralization activity of AF. We did not detect viral antigen in either early or late

term AF samples even though corresponding fetuses were infected. Boudreaux *et al* (2009), reported 12 of 14 (86%) infected fetuses from FIV- infected queens at early gestation, using real time RT-PCR to detect viral RNA. Using standard PCR to detect FIV provirus and serology to detect FIV-specific antibody, Weaver et al (2005) found 21 of 22 (95%) infected fetuses at late gestation. Corresponding placentas also were positive (Boudreaux *et al.*, 2009; Weaver *et al.*, 2005). These data indicate that either virus did not enter the AF fluid during gestation, or that the viral load was below the limit of detection. Our data are in agreement with those of Rogers and Hoover (2002) who were unable to detect FIV RNA in AF from full-term pregnancies in FIV-infected queens. We collected the AF by needle aspiration prior to opening the fetal membranes, so there was no possibility of external contamination with maternal blood. Exposure of the fetus to AF contaminated with maternal blood may occur following prolonged membrane rupture, providing a means of perinatal HIV infection (Kind *et al.*, 1998). In contrast, HIV-1 was previously isolated from the AF at 15 week gestation and 38 week gestation (Mundy *et al.*, 1987; Sprecher S, 1986). Jaspan et al (2004) detected approximately 5-10 pg/100 µl of HIV-1 p24 antigen in 8 of 10 AF samples collected from full term HIV-infected women, all of whom had been treated with AZT during months of pregnancy and during labor and delivery.

Despite the fact that all maternal serum samples were FIV seropositive within 4 weeks p.i. using SNAP ELISA and Western blotting, using the PetChek FIV Antibody Test Kit ELISA to quantify antibody titer, only 2 of the 6 sera (animals 8035 and 5111) were positive. Two bleed dates were evaluated using

this assay, week 8 p.i. and delivery, and the levels of antibody in these two cats were higher at the earlier time point. The conflicting serological results indicate that both the SNAP ELISA and Western blot were more sensitive assays than the PetChek ELISA. Using a commercial ELISA produced by the same company (IDEXX), Baldinotti et. al (1994) detected FIV-specific antibody reactions in the sera of cats infected with FIV-M2 (Pisa-M2) and FIV-Pet (Petaluma strain) collected between 1 and 28 months p.i. Thus, the FIV antibody PetChek ELISA may have strain-specific sensitivity. The FIV isolate(s) used to prepare antigen for this kit are unknown to us due to proprietary rights of the manufacturer.

Jaspan et al (2004) compared antibody levels in paired AF and plasma from SIV-infected rhesus macaques and HIV-1-infected patients. Although plasma normally has higher immunoglobulin content than AF, they found HIV-1 specific antibodies to be 20 to 700-fold higher in AF after adjusting for concentration differences. Likewise, SIV p27-specific antibody was higher in AF than in plasma in macaques, after concentration adjustments were made. We found the same pattern in the FIV-infected cat at early gestation. Without adjusting for naturally-higher concentration of total immunoglobulin in plasma, we found at least one AF from five of six queens to have FIV-specific antibody levels (based on S/P ratios) ranging from approximately 4 to > 21-fold higher than the corresponding serum. The two AFs from queen 8035 were both negative for antibody. Interestingly, these antibody-negative AF were obtained from fetal membranes of fetuses 8035A and B; both fetuses were negative for viral RNA, while their placentas were FIV RNA positive (Boudreaux *et al.*, 2009). Cat 8035

did, however, produce one FIV-infected fetus (8035C), but the AF from that fetus was not tested in this study. The disparate immunoreactivity that was detected in different AF from the same queen shows the usefulness of this feline model in studying fetus-specific pathology without the confounding maternal variables that naturally arise between individuals in human and non-human primates infected with their respective immunodeficiency viruses.

Using a syncytium-reduction assay, virus neutralization capability was detected in three of three AF samples collected from FIV-infected queens at late gestation, while only one of the corresponding maternal serum samples (6062) neutralized the virus. While FIV-specific antibody was not detected in serum from any of the three early-term queens using PetChek ELISA, all animals were strongly positive by Western blot at corresponding times p.i. However, seroreactivity to envelope glycoproteins, which express the major neutralizing epitopes, were not measured by either ELISA or Western blot, so information gained from these assays is limited. The discrepancy in neutralization between AF and sera likely corresponds to the greater concentration of FIV-specific antibody in AF. The same assay failed to detect virus neutralization capability in AF from any of three FIV-infected queens at late gestation. While the reason for differences in neutralization capability between early and late gestation is unknown, AF samples had been stored at -20°C for more than five years, and some antibody may have degraded. Both sets of animals (early gestation and late gestation) were infected with a plasma pool obtained from three cats that were experimentally-infected with FIV-B-2542, rather than cloned virus. Thus,

antigenic drift, resulting from viral evolution in multiple hosts, may have generated variation in the immunodominant neutralization epitopes and consequent variability in dominant neutralizing antibodies. Neutralization escape mutants evolve frequently during the course of lentiviral infection as a result of the selective pressure applied by the humoral immune response (Burns & Desrosiers, 1994; Hussain *et al.*, 1987; Zheng *et al.*, 1997). The FIV-B-2542 plasma pool used to infect CrFK cells in the syncytium reduction assays had been passed through fewer animals. Therefore, predominant neutralizing antibodies in both AF and sera may have been directed towards more recently-evolved variants, diminishing the neutralizing capability of our samples. Others reported that passage history clearly effects serum neutralization capability in FIV-infected cats and that poor neutralizing antibody responses are generated in either natural or experimental infection (Baldinotti *et al.*, 1994). On the other hand, broadly neutralizing antibodies were identified in some patients who had been infected with HIV-1 for more than 9 years (Parren *et al.*, 1998).

The role that maternal neutralizing antibodies may play in preventing HIV transmission to infants is unresolved. While maternal autologous neutralizing antibodies can be highly effective in preventing MTCT, variants that escape these maternal antibodies can infect the fetus (Bal *et al.*, 1996; Dickover *et al.*, 2006). Others also report that high titers of neutralizing antibodies did not prevent fetal infection (Bal *et al.*, 1996), and low maternal neutralizing antibody did not increase the risk of transmission in women matched for CD4+T cell percentage (Husson *et al.*, 1995). Typically, the fetus is infected with a

genetically homogeneous, non-syncytium-inducing, macrophage-tropic strain selected from the maternal quasispecies (Reinhardt *et al.*, 1994).

It has been speculated that serum factors other than antibodies may be responsible for virus neutralization (Lunardi-Iskandar *et al.*, 1998). Bradley *et*. *al* (1982) found that depletion of IgG from amniotic fluids eliminated virus neutralization capability of the AF, concluding that IgG antibodies were the sole source of virus neutralization. We speculated that a possible blood component that could contribute to reduced viral infectivity is complement. Virus-antibody interactions could activate complement activity within the AF. However, we eliminated potential antiviral action of complement by heat inactivating AF samples prior to use in syncytium reduction assays.

In summary, the FIV load was either negligible or below the level of detection in all AF collected at early or late gestation, and thus, direct infection of the fetus by exposure to contaminated AF seems unlikely. FIV-specific antibody was commonly present in AF samples, although AF from different fetuses had different amounts of antibody, or none at all. Virus-neutralizing antibodies were detected in the three early-gestation AF tested from the FIV-infected group, while virus neutralization did not reach the level of significance among the late-term AF samples. The presence of VN antibodies in AF did not correlate with fetal infection. Despite the presence of VN antibodies in AF, the corresponding early-term fetuses were infected with FIV. Collectively, these data suggest that the amniotic fluid does not play a significant role in in utero FIV infection.

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CHAPTER III

SUMMARY

In the U.S., 99% of HIV-infected children were infected due to MTCT (CDC, 2007b). In the absence of administration of anti-retroviral therapy to HIV-infected, pregnant women, the rate of MTCT is 15-40% (Brocklehurst & Volmink, 2002; Burns & Mofenson, 1999; Connor *et al.*, 1994; Jaspan & Garry, 2003; Newell, 1998; Simpson *et al.*, 2000). However, between 1994 and 2007, aggressive anti-retroviral therapy and cesarean delivery resulted in an estimated 96% decrease in perinatal transmission (CDC, 2007a). Maternal virological and serological factors such as viral load, virus strain, and CD4+ T cell counts, are predictors of MTCT (Deeks *et al.*, 2004; McCune, 2001; Yates *et al.*, 2007). HIV- and SIV-contaminated amniotic fluids were detected, and virus neutralizing antibodies were found in AF of both humans and macaques. Virus neutralizing antibody concentrations were significantly higher in the AF than plasma of HIV-infected humans or SIV-infected macaques (Jaspan *et al.*, 2004). Yet, a direct relationship between the presence or absence of HIV or virus neutralizing antibodies in AF has not been resolved. HIV-1-infected gastric aspirates were reported in neonates (Mandelbrot *et al.*, 1999). Thus, a role for AF in promoting or limiting perinatal transmission remains controversial.

Using the FIV-infected cat to model lentiviral MTCT, we reported very high fetal infection rates in both early and late, cesarean section-delivered fetuses (Boudreaux *et al.*, 2009; Weaver *et al.*, 2005). The apparent frequency of vertical transfer in experimentally-infected cats and the fact that cats produce multiple offspring in a single litter (limiting the confounding variables innate to individual queens) makes this animal model useful for evaluating the role of AF in MTCT. We suspected that contaminated AF may provide a source of fetal infection, but also predicted that virus neutralizing antibodies, potentially present in AF, could limit this means of vertical transmission. Our goals were to determine whether FIV antigen is present in AF, to analyze AF and corresponding sera for FIV p24-specific antibodies, and to determine whether AF and sera had virus neutralizing activity.

We used both real time RT-PCR to detect viral RNA and an FIV p24-capture ELISA to look for the presence of FIV in the amniotic fluid associated with early- and late-term fetuses. We were unable to detect the virus by either assay method, indicating the virus was either completely absent or at a level beneath the limit of detection. We also evaluated the AF for FIV p24-specific antibody using two different ELISA techniques. We detected anti-FIV p24 antibody in most early- and late-term AF (63% and 61%, respectively). Corresponding early- and late- term serum samples were also positive for the FIV-specific antibody by Western blot and SNAP ELISA. However, the 96 well PetChek FIV Antibody Test kit, used to test the early sera only, was less sensitive (33% positive).

After determining that FIV-specific antibody was present in AF, we evaluated the virus neutralization capability of randomly- selected AF samples and corresponding serum from early pregnancies by conducting in vitro syncytium-reduction assays. These assays involved incubating triplicate cultures of FIV- infected CrFK cells with AF or serum diluted in a 4-fold series or medium lacking either fluid. Following incubation of the cultures for 1 week, all wells were stained and observed microscopically for the development of syncytia. All syncytia within the triplicate cultures were counted, averaged, and analyzed statistically. Virus neutralization by the fluids was evident by a reduction in the number of syncytia. All early-term AF had virus neutralizing activity. One serum sample neutralized the virus. None of the AF samples collected from late-gestation fetal membranes reduced syncytia to a level that reached significance. It is possible that long-term storage diminished the antibody quality in late-term samples as they were preserved at -20°C for more than 5 years. Virus neutralization assays for corresponding late-term sera were not done.

In summary, we failed to detect FIV in AF, suggesting that virus was not present in these fluids. We detected FIV-p24 specific antibody in the majority of AF from both early and late pregnancy. We observed virus neutralizing activity in three of three early-term AF and one corresponding serum, but not late-term AF. We observed a very high rate of fetal infection at both stages of pregnancy. Thus, it appears that amniotic fluids play no appreciable role in promoting or limiting vertical transfer of FIV.

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