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Maternal Hematological and Virological Characteristics during Early Feline Immunodeficiency Virus (FIV) Infection of Cats as Predictors of Fetal Infection and Reproductive Outcome at Early Gestation

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Abstract

The FIV-infected cat is a small animal model for HIV mother-to-child transmission (MTCT) because the two lentiviruses are biologically related and produce similar clinical syndromes. Both viruses are vertically transmissible and may negatively impact reproductive outcome. Maternal hematological and virological parameters are predictors of MTCT in HIV-infected women. Our purpose was to determine whether similar maternal characteristics during early pregnancy in FIV-infected cats influence pregnancy outcome. We inoculated ten cats with FIV-B-2542; 10 cats were uninoculated. We quantified longitudinal CD4:CD8 T cell ratios, proviral load, and plasma viremia, monitored longitudinal serostatus, and documented clinical and reproductive outcome during early pregnancy. Inoculated queens were seropositive and provirus positive by week 4 post infection (p.i.). CD4:CD8 ratios were depressed in the infected group by month 3.5 p.i. Proviral load was variable in the animals throughout the course of infection; plasma viremia was below the level of detection in all animals. Reduced litter sizes and increased fetal demise occurred in infected queens. Viral RNA, but not proviral DNA, was detected in representative placentas (14 of 14; 100%) and fetuses (12 of 14; 86%) collected from infected queens. However, maternal virological and hematological characteristics did not correlate either positively or negatively with reproductive outcome.

Keywords

Feline Immunodeficiency Virus; Mother-to-child Transmission; Pediatric AIDS; CD4:CD8 T Cell Ratio; Provirus Load; Plasma Viremia

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1. Introduction

Globally, more than 420,000 children were newly infected with HIV in 2007, representing 16% of new HIV infections (UNAIDS, 2008). MTCT accounts for more than 90% of pediatric infections (CDC, 2008). In addition, HIV infection of pregnant women often results in poor outcome, including low-birth-weight babies, preterm delivery, and enhanced incidence of spontaneous abortions (D'Ubaldo et al., 1998; Kumar et al., 1995; Langston et al., 1995).

Maternal virological and hematological factors including plasma viremia and CD4⁺ T cell counts influence HIV vertical transfer. MTCT typically accompanies a decrease in the maternal CD4⁺ T cell population, resulting from virus-mediated destruction of these cells (Deeks et al., 2004; McCune, 2001; Yates et al., 2007), and high maternal plasma virus load (O'Donovan et al., 2000).

The FIV-infected cat provides an excellent small animal model of HIV MTCT because the viruses share many common genetic and biological features and produce very similar clinical syndromes in their respective hosts (Willett et al., 1997). FIV is readily transmitted from mother-to-child under experimental conditions, resulting in pregnancy outcomes similar to those of HIV-infected pregnant women.

A high rate of vertical transmission of FIV occurs late in gestation in experimentally-infected cats with frequent reproductive failure (Allison and Hoover, 2003a; O'Neil et al., 1996; Rogers and Hoover, 1998, 2002; Weaver et al., 2005). We hypothesized that maternal virological and hematological characteristics occurring during early pregnancy in the FIV-infected cat may be predictive of pregnancy outcome. Our objectives were to: 1) quantify T cell population dynamics occurring in the peripheral circulation of queens during early FIV infection; 2) confirm and quantify FIV infection in longitudinal blood samples; and 3) determine virus-induced reproductive outcome during early pregnancy. We report reduced fecundity and increased fetal loss during early gestation in the infected group. Viral RNA, but not provirus, was detected in placentas and fetuses. The CD4:CD8 T cell ratio declined significantly in the infected group within 3.5 months p.i. However, individual CD4:CD8 T cells ratios neither positively nor negatively correlated with pregnancy outcome. Plasma viremia was below detectable limits at all time points in all cats, but cats were provirus positive and seropositive within four weeks p.i. and remained positive throughout the duration of the experiment. Maternal hematological and virological correlates of reproductive outcome were not clearly identified in this study.

2. Materials and Methods

2.1 Animals and virus

Cats were female, reproductively mature, specific pathogen-free (SPF) animals (*Felis domesticus*), obtained from a commercial cattery. Physical evaluation of cats, including respiration, pulse rate, weight, and body condition, was done two weeks prior to inoculation by trained veterinary staff. Ten cats were inoculated intravenously with 1 cc of a feline plasma pool containing approximately 1×10^4 copies per ml of FIV-B-2542 (Weaver et al., 2005); ten cats were uninoculated controls. All animals were evaluated by caretakers on a daily basis, and veterinary care was administered appropriately. Whole blood (15 ml) was collected into Vacutainer® tubes at biweekly to monthly intervals until delivery for the collection of serum, plasma, and peripheral blood leukocytes (PBLs). Following confirmation of infection by PCR and serology, described below, queens were allowed to breed naturally with SPF males. The time of FIV inoculation until delivery ranged from approximately 9.5 months to 13.5 months (mean 11.14 months). Fetuses were delivered by cesarean section immediately after pregnancies were confirmed by ultrasonography, at week 3–4 gestation. Infected queens were

euthanized following delivery using Beuthanasia at 1 cc per 10 lb body weight. Control cats were spayed and released for adoption after recovery. Fetuses, placentas, and amniotic fluids were collected from all animals. Tissues were snap frozen in liquid nitrogen or fixed in formalin. Tissues and amniotic fluids were frozen at -80°C . Animal protocols were approved by the Institutional Animal Care and Use Committee of Mississippi State University.

2.2 Flow cytometric analysis of CD4+ and CD8+ T cell populations

Mouse anti-feline monoclonal antibodies to CD4 and CD8 conjugated with phycoerythrin (PE) and fluorescein isothiocyanate (FITC) (Southern Biotech, Birmingham, AL), respectively, were diluted 1:20 in PBS. Whole blood was sampled at 250 μl , and red cells were lysed with ACK lysing buffer (Quality Biological, Inc., Gaithersburg, MD). All samples were washed with PBS and centrifuged at $400 \times g$ for 5 min using a Beckman TJ-6 centrifuge. Samples were double stained with 40 μl of each diluted antibody and incubated 15 min at room temperature in the dark. Flow cytometry was performed using FACSCalibur (BD Biosciences, San Jose, CA); 10,000 events were collected.

2.3 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 $11,750 \times g$ in a GSA centrifuge at 4°C for 20 min to remove cellular debris. The clarified supernatant was centrifuged at $23,000 \times g$ 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 μl of PBS and stored at -80°C . Real time reverse transcriptase (RT)-PCR, described below, was used to confirm FIV RNA in these preparations.

2.4 SDS-PAGE and Western blot

Viral proteins from the purified virus preparations were resolved under reducing conditions on 12% SDS-polyacrylamide gels. Electrophoresis was done using a Mini Protein II system (Bio-Rad Laboratories, Hercules, CA), according to standard procedure (Laemmli, 1970).

Proteins were trans-blotted onto nitrocellulose membranes (Micron Separations Inc., Westborough, MA) using the Trans Blot SD Semi-Dry Transfer cell (Bio-Rad) and Towbin buffer containing methanol (25mM Tris, 192mM glycine, 20% methanol, pH 8.3). The nitrocellulose was cut into vertical strips for use in Western blots.

Longitudinal serum samples (diluted 1:50) were evaluated by Western blot according to established protocol (Whetstone, 1991). The secondary antibody (diluted 1:1000) was goat anti-cat IgG peroxidase (MP Biomedicals, Solon, OH). Blots were developed using a solution of 0.02 g 4-chloronaphthol dissolved in 5 ml of methanol, 25 ml 0.05 M Tris (pH 6.8), and 12 μl hydrogen peroxide. Positive and negative control sera, obtained from FIV-B-2542 inoculated and control cats from a previous study (Weaver et al., 2005), were included in all Western blot assays.

2.5 ELISA to detect FIV-specific antibody in serum

Longitudinal sera from the inoculated queens were evaluated for seroconversion using the SNAP FIV/FeLV Combo Test Kit (IDEXX Laboratories, Westbrook, ME), according to kit instructions. Sera collected at week 2 p.i and thereafter were evaluated until seroconversion was detected.

2.6 ELISA to detect FIV p24 antigen in plasma

Plasma samples collected at three different time points p.i. were evaluated for FIV p24 antigen using the Petcheck Feline Immunodeficiency Virus Antigen Test Kit (IDEXX Laboratories), according to kit instructions. Absorbance values at 650 nm (A_{650}) for plasma samples and positive and negative controls (supplied in kit) were measured using a plate reader. The cutoff value was calculated based on mean values for negative controls. Samples with A_{650} values below the cutoff value were considered negative.

2.7 Purification of DNA and RNA from feline plasma, PBLs, and tissues

TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to fractionate RNA and DNA from PBLs and sections of placental and fetal tissues. RNA was purified from the aqueous phase as described (Scott et al., 2008). DNA was further purified from the organic phase using the DNEasy Kit (Qiagen, Valencia, CA). RNA was isolated from plasma using the QIAampViral RNA Mini Kit (Qiagen). DNA and RNA concentrations were determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA) and frozen at -80°C . Viral RNA to be used as standard was isolated from purified, whole virus, using the QIAamp Viral RNA Mini Kit (Qiagen), treated with RNase-free DNase, and quantified to estimate copy number.

2.8 Detection of provirus in PBLs and placental and fetal tissue using PCR

FIV provirus was amplified from PBLs and feline tissues using standard, nested PCR targeting a 293 bp region of the FIV gag gene (Allison and Hoover, 2003a, b). DNA obtained from FIV-B-2542 infected MCH 5-4 cells was used as a positive control in PCR analyses. Reaction conditions were reported (Weaver et al., 2005). The limit of detection was previously determined to be 1 to 10 copies of target (Allison and Hoover, 2003a, b).

2.9 Quantification of FIV provirus by limiting dilution PCR

A two-fold dilution series of DNA isolated from PBLs at three time points p.i. was done to determine the end point dilution for detection of FIV provirus, using PCR to detect the 293 bp gag proviral fragment. It was assumed that the highest positive dilution of DNA contained at least one copy of provirus. Virus load was estimated as copies per microgram of DNA.

2.10 Detection of FIV RNA in placentas, fetuses, and maternal plasma using TaqMan real time RT-PCR

Real time TaqMan Primers and probes targeting the FIV gag gene and β -actin were obtained commercially (MWG-BIOTECH, Inc., High Point, NC). Sequences (Scott et al., 2008; Weaver et al., 2005) and 5' and 3' probe labels (Scott et al., 2008) were previously reported. The real-time RT-PCR, targeting FIV gag in placental, fetal, and plasma RNA, was done using an iCycler (Bio-Rad) according to established parameters, and normalized Ct values were determined (Scott et al., 2008). To determine the limit of detection, five-fold dilutions of the RNA standard in DEPC-treated water (ranging from 1:5 to $1:1.95 \times 10^6$) were subjected to the real time RT-PCR protocol to achieve a standard curve. The limit of detection was 2×10^4 copies/ μl RNA.

2.11 Statistical analyses

Statistical evaluation of T cell populations was done using single-factor ANOVA (Microsoft Excel-XP, Redmond, WA).

3. Results and Discussion

Routine physical examination of control queens began at their arrival and continued throughout the breeding period, which ranged from 5 months to 9.5 months (mean 7 months) after

experimentation began. No noteworthy abnormalities were revealed. Several abnormalities were noted among the FIV-infected group. Within approximately 6 weeks p.i., cat 6850 developed sudden dehydration and fever which was unresponsive to antibiotic therapy. The cat was euthanized at month 2 p.i. Cat 0326 developed conjunctivitis at month 3 p.i. which responded well to antibiotic treatment. Three cats, 6062, 5587, and 3779 developed reproductive problems, discussed below.

Maternal hematological and virological characteristics are known to influence maternal-fetal transmission of HIV. High levels of plasma viremia and depressed CD4⁺ T cells positively correlate with fetal infection (O'Donovan et al., 2000). We evaluated these predictors of early in utero transmission during the first year of FIV infection in the cat model of MTCT.

For both control and infected groups, the mean percentages of CD4⁺ and CD8⁺ T cells were determined at each time point sampled, and CD4:CD8 T cell ratios were calculated. Longitudinal CD4:CD8 ratios for individual infected cats are shown in Figure 1. The CD4:CD8 ratio for the control group was calculated from the collective CD4⁺ and CD8⁺ T cell percentages of all control cats measured. With the exception of cat 5111, whose CD4:CD8 ratio increased, FIV infection caused a decreased T cell ratio in all cats. However, T cell dynamics were not predictive of pregnancy outcome. The mean CD4:CD8 ratio was significantly depressed in the infected group by month 3.5 p.i. ($P=0.03$), and it remained depressed at later sampling intervals. The decreased T cell ratio was a result of depletion of the CD4⁺ T cell population in the infected group by month 3.5 p.i., while no significant changes were observed in the CD8⁺ T cells in the infected group.

The mean CD4:CD8 T cell ratio determined for control cats in this study was 2.7, falling within the normal range for cats (Dean et al., 1991; Novotney et al., 1990). In a previous study, the CD4:CD8 T cell ratio in peripheral blood dropped below 1.0 by twenty weeks of FIV infection and remained low throughout the course of infection (Song et al., 1992). The depletion in the CD4⁺ T cell population was explained in part by the virus's preferential infection of activated CD4⁺ T cells, which most abundantly express CD134.

By week 4 p.i. all animals were weakly seropositive for FIV p24-specific antibody, as determined by ELISA (Table 1). Nearly all cats remained seropositive with increasing intensity of seroreactivity at subsequent time points until termination/delivery (Figure 2). FIV provirus was detected in PBLs of all infected animals at week 4 p.i. Animals remained provirus positive throughout the duration of the study (Figure 2).

Proviral loads, estimated as copy number per microgram of DNA, were determined at months 1 and 3 p.i. and at delivery and/or euthanasia (Figure 3A). By 1 month p.i., proviral loads varied among infected queens, ranging from a low of 80 copies/ μ g DNA to greater than 3900 copies/ μ g DNA.

Interestingly, the proviral load of cat 6850, euthanized due to unresolvable infection at month 2 p.i., was lowest of any cat at 1 month p.i. but increased more than any other animal, a 41-fold increase by month 2 p.i. (Figure 3A). This increase in proviral load represented a 7-fold greater increase than in the cat producing the next highest increase in provirus.

Proviral copy number in peripheral blood was neither positively nor negatively correlated with pregnancy outcome. Queen 1893 produced the largest litter size, 5 viable fetuses and 1 non-viable fetus (Table 1), yet this animal had the highest proviral load (> 2200 copies/ μ g DNA) of any reproductively-successful animal (Figure 3A). On the other hand, queen 8035 produced the greatest amount of fetal damage (3 non-viable fetuses; 1 viable fetus) (Table 1), but this animal had among the lowest terminal proviral loads (Figure 3A).

Two animals who failed to conceive, cats 5587 and 3779, had among the highest proviral loads (> 1000 copies/ μ g DNA) at the time of euthanasia (Figure 3A). While it is tempting to speculate that FIV infection caused the infertility of these two cats, reproductive tissues from these two cats were not evaluated for viral infection. Moreover, two control cats also failed to conceive after repeated attempts at breeding. Consequently, the role that FIV infection may have played in their reproductive pathologies is unclear.

The variability in proviral load that we observed is not uncommon in FIV infection. Fluctuation in proviral loads during the course of infection due to a decrease in infected CD4⁺ T cells was reported by others who determined that CD4⁺ T cell depletion occurred as a result of targeting by cytolytic T lymphocytes and apoptosis (Ohkura et al., 1997).

Using real time TaqMan RT-PCR targeting FIV gag RNA, fourteen representative placentas and their corresponding fetuses were evaluated for viral infection (Figure 3B and C). With the exception of queen 0326, who produced only one offspring, two to three placentas per queen were evaluated (Figure 3B). Fourteen of fourteen placentas (100%) and twelve of fourteen fetuses (86%) were positive. A comparison of mean Ct values provided a means to determine relative gene expression from each sample. Negative control tissues yielded no fluorescent signal (Ct = 0); positive control tissue yielded a mean Ct value of 27. Twelve of fourteen placentas were strongly positive (Ct ≤ 30), while two placentas (5111 B, 1126 A) gave a moderate reaction (Ct 31.4 and 30.1, respectively). On the other hand, only three fetal specimens were strongly positive (8035 C, 0866 A, and 0866 B) (Figure 3C). The remaining samples were either moderately to weakly positive (Ct ≥ 30) or negative (8035 A and B). It was interesting to note that two of three placentas from cat 8035 (A and B) were negative, while the third placenta (8035 C) was strongly positive. Samples B and C from this animal were placentas corresponding to arrested fetuses, while sample A came from a viable fetus. Also, fetus 0866 A was viable while 0866 B was a resorption. Both of these fetal samples were strong positives. While copy number was not quantified in these samples, these data suggest that the virus load in placental or fetal tissue did not determine pregnancy outcome.

Proviral DNA was not detected in placental or fetal tissues using standard, nested PCR analysis. These findings indicate that our calculated real time RT-PCR detection limit (2×10^4 copies/ μ l) is an underestimation of assay sensitivity, probably due to co-purification of cellular RNA in our viral RNA standard, which confounds calculation of viral copy number. Others (Rogers and Hoover, 1998) were unable to detect fetal infection prior to 5 weeks of gestation but detected infection in 96% of placentas at early, intermediate, and late stages of gestation, using standard, nested PCR. MTCT occurring in HIV-infected women is most frequent during late stages of pregnancy and at delivery (Brossard et al., 1995; Chouquet et al., 1999).

FIV p24 antigen was not detected in any of the plasma samples collected from infected cats at any time point p.i. Likewise, viral RNA was not amplified from plasma samples from infected cats at any time period. Spiking plasma with standard viral RNA resulted in amplification, proving that reaction conditions were appropriate. Thus, plasma viremia was below the level of detection in all animals. Maternal plasma viral RNA loads were measured previously in FIV-B-2542-infected queens at 3, 5, 7, and 9 weeks pregnancy. Plasma viral RNA loads below the detection threshold (1000 copies/ml) occurred in nearly half the queens, regardless of pregnancy stage, and only one animal had a plasma viral RNA load above the threshold value (Rogers and Hoover, 1998).

Pregnancy outcome in individual control and infected queens is reported (Table 1). Eight control cats produced 41 viable and 2 non-viable fetuses (4.7% non-viability), an average of 5.4 offspring/litter. Two control animals failed to conceive. An undiagnosed uterine abnormality was noted upon ultrasonography of one of these animals (4102). The other cat,

9307, showed no evidence of reproductive pathology. Both of these animals were otherwise clinically normal. Seven infected queens produced 21 viable and 6 non-viable fetuses (22.2% non-viability), an average of 3.9 offspring/litter. Infected cats 5587 and 3779 failed to conceive after repeated matings, due to pyometra and ovarian cysts, respectively. Cat 6062, who produced 3 viable fetuses in the left uterine horn, had right horn pyometra at delivery. The difference in the number of viable fetuses between the infected and control groups, nearly 50% fewer in infected cats, was significant ($P=0.02$). Although three times higher in the infected group, fetal non-viability failed to reach the traditional level of significance ($P>0.05$). High rates of fetal non-viability in full or near-full term FIV-infected cats were reported elsewhere (O'Neil et al., 1995; Weaver et al., 2005), but rates of fetal demise in FIV-infected cats at this early stage of pregnancy have not been reported.

These same maternal hematological and virological characteristics were not evaluated by us at late pregnancy, where high rates of fetal infection and increased reproductive failure occurred (Weaver et al., 2005). However, other investigators found that these maternal markers did not change significantly with gestational age, although MTCT correspondingly increased (Rogers and Hoover, 1998).

In summary, maternal correlates of HIV vertical transmission, including peripheral blood provirus load, plasma viremia, and depressed CD4+ T cell populations, neither positively nor negatively correlated with fetal infection or pregnancy outcome in the FIV-infected cat model at 3–4 weeks gestation. Our data, coupled with those reported previously (Rogers and Hoover, 1998), indicate that the experimentally-infected cat may not adequately model these particular maternal correlates of HIV MTCT. However, it is clear that early FIV infection reduced fecundity, compromised reproductive outcome, and resulted in a high rate of MTCT during early weeks of pregnancy. The inability to correlate fetal infection with reproductive outcome suggests an indirect mechanism of fetal damage, such as virus-induced placental pathology or pathology of other reproductive tissues. We previously reported preliminary evidence for a role for placental inflammation in compromised pregnancy in the FIV-infected cat (Scott et al., 2008; Weaver et al., 2005). The effect of FIV infection on placental immune parameters at early and late pregnancy is currently ongoing.

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Figure 1.

CD4:CD8 T cell ratios in longitudinal blood samples from FIV-infected cats. The mean CD4:CD8 ratio for control cats, determined by compiling data from all sample periods, is included for comparison (dashed line at 2.75 flanked by the standard error of the mean, shaded in grey). Individual cat numbers are shown in the legend at right. CD4+ and CD8+ T cells were quantified from whole blood samples collected at regular times points p.i. The data are plotted beginning at 1 month p.i. and representative time points are shown through termination. Although the T cell ratios fluctuated in individual animals over time, by delivery and/or euthanasia at 9 to 12 months p.i., a decline in the CD4:CD8 T cell ratio had occurred in all animals except cat 5111. CD4:CD8 ratios were significantly different from the control by 3.5 months p.i. and at subsequent time points ($P < 0.05$).

Figure 2.

Detection of FIV provirus and FIV p24-specific antibodies in longitudinal blood samples from FIV infected cats. Left panels: FIV provirus was detected by standard PCR targeting a 293 bp region of the gag gene. PCR products were resolved on 1% agarose gels. By week 4 week p.i., the virus was detected in all infected animals. (+) shows the PCR product amplified from DNA purified from cultured MCH 5-4 cells infected with FIV-B-2542. Right Panels: Western blot analysis of longitudinal serum samples (diluted 1:50) was performed following resolution of purified FIV proteins on 12% SDS-PAGE gels and transfer to nitrocellulose membranes. Positive and negative controls (not shown) were sera obtained from FIV infected and uninfected cats from a previous study. FIV p24 is the major immunodominant viral protein, and seroreactivity was most consistent toward this protein. Seroconversion was detected by week 4 p.i. and persisted until termination.

Figure 3.

Determination of FIV proviral copy number in longitudinal blood samples and relative expression of viral RNA in placentas and fetuses from FIV infected cats. (A) Proviral copy number was determined using limiting dilution PCR targeting a 293 bp region of the FIV gag gene. Proviral load is reported as copies/µg DNA at 1 and 3 months p.i. and terminal time points. (B) Placental and (C) fetal expression of FIV RNA was determined using TaqMan real time RT-PCR targeting the FIV gag gene. Mean Ct values were determined from duplicate samples. Ct values are inversely related to viral RNA load. Placental or fetal RNA from two uninoculated cats (6108 and 9276) were included as negative controls. Mean positive control

(striped bar) represents mean Ct values from 10 placental samples from 6 FIV positive cats from a previous study.

Table 1
Maternal Hematology, Virology, and Pregnancy Outcome in Control and Infected Queens

Cat number	FIV Serostatus*	Plasma Viremia**	CD4:CD8 Ratio (Terminal)	Viable Fetuses	Non-viable Fetuses	Reproductive complications
3550	-	nt	2.19	4	0	
6108	-	nt	2.68	4	0	
7824	-	nt	1.91	4	0	
8291	-	nt	1.44	4	0	
8059	-	nt	3.90	6	0	
2779	-	nt	2.29	7	1	
5373	-	nt	3.30	7	0	
9276	-	nt	1.82	5	1	
4102	-	nt	3.25	0	0	Unknown uterine abnormality
9307	-	nt	2.70	0	0	Failed to conceive
Total Fetuses				41	2	4.7% non-viability
0326	+	-	2.09	1	0	
8035	+	-	1.43	1	3	
5111	+	-	2.57	2	1	
0866	+	-	0.95	5	1	
1893	+	-	1.39	5	1	
6062	+	-	0.37	3	0	Right horn pyometra
1126	+	-	0.43	4	0	
5587	+	-	0.84	0	0	Pyometra
3779	+	-	1.18	0	0	Ovarian cysts
6850	+	-	N/A	0	0	Euthanized before mating
Total Fetuses				21	6	22.2% non-viability

* Sera from all inoculated queens were tested by ELISA and Western blot; all queens were positive by week 4 p.i.

** Plasma was tested by real time RT-PCR for viral RNA and by FIV p24 ELISA for viral antigen.

nt = Not tested