DISSERTATION

FELINE LEUKEMIA VIRUS INFECTION: A MODEL OF EFFECTIVE RETROVIRAL IMMUNITY

Submitted by

Andrea Nicole Torres

Department of Microbiology, Immunology, and Pathology

In partial fulfillment of the requirements

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ANDREA NICOLE TORRES ENTITLED FELINE LEUKEMIA VIRUS INFECTION: A MODEL OF EFFECTIVE RETROVIRAL IMMUNITY BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Advisor

Department head

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ABSTRACT OF DISSERTATION

FELINE LEUKEMIA VIRUS INFECTION: A MODEL OF EFFECTIVE RETROVIRAL IMMUNITY

Feline leukemia virus (FeLV) infection is a naturally occurring, horizontally transmitted gammaretrovirus infection of cats that causes cancer and immunosuppression. A substantial fraction of cats exposed to FeLV effectively contain virus and resist induction of persistent antigenemia and viremia. Little is known, however, about the state of the viral genome in these animals. To further explore FeLV:host relationships using more sensitive nucleic acid-based detection methods, we developed a quantitative real-time PCR (qPCR) assay to detect FeLV DNA in blood cells and tissues. This assay allowed us to detect DNA in circulating cells of non-antigenemic cats that had ostensibly resisted viral challenge. Thus, we categorized four FeLV:host relationships in cats exposed to FeLV: 1) undetectable antigenemia and viral DNA; 2) undetectable antigenemia but low viral DNA levels; 3) transient antigenemia and moderate viral DNA levels; and 4) persistent antigenemia and high viral DNA levels. These host:virus relationships were established by 8 weeks post-challenge and were maintained for years. Because viral DNA levels in circulation and tissues were highly correlated, we surmise that exposed cats in which viral DNA was not detectable did not maintain a tissue reservoir. We next sought to determine whether the viral DNA detected was

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transcriptionally active. Thereby a reverse transcriptase qPCR assay was developed to quantitate extracellular FeLV RNA. We demonstrated an almost perfect agreement and a strong linear relationship between viral DNA and RNA levels, inferring that detected FeLV DNA is both integrated and transcriptionally active. However, only high levels of viral DNA and RNA were associated with detectable infectious virus. Finally, these studies also demonstrated that two whole inactivated virus (WIV) adjuvanted FeLV vaccines provided effective protection against FeLV challenge. In nearly every recipient of these vaccines, neither viral DNA, RNA, antigen, nor infectious virus could be detected in blood. Moreover, effective viral containment occurred despite a weak virus neutralizing antibody response. The above findings extend and reinforce the precept of FeLV infection as a model of the early immune responses that determine effective vs. ineffective containment of retroviral infections, and hold valuable insights into immunoprevention and therapy.

Andrea Nicole Torres
Department of Microbiology,
Immunology, and Pathology
Colorado State University
Fort Collins, CO 80523
Fall 2007

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In conclusion, I want to pay tribute to the cats. I salute you.

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DEDICATION

I dedicate this dissertation to my family. My dear husband and best friend Matty has supported me through this journey with love and laughter and his belief that I would succeed during all the times I believed I would not. My precious girls Maya Sol and Emma Celiana have given me perspective and have taught me a simplified scientific method: slow down, take awe at this world, and ask why. To my budding scientists -- girls can do anything!

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INTRODUCTION

Identification:

Feline leukemia virus (FeLV) was first identified as a naturally occurring viral infection of cats more than 40 years ago when Jarrett *et al.* were investigating clusters of lymphoma cases in multi-cat households (51, 52). Distinctive crescent-shaped nascent nucleocapsids referred to as 'C-type' virus particles were visualized by electron microscopy as cell-free in the plasma of cats with leukemia and budding from the membranes of malignant lymphoblasts from a cat with naturally occurring lymphocytic leukemia (54, 99). These virions were approximately 110 nm in diameter and morphologically similar to murine leukemia virus (54, 100). Subsequently the horizontal transmission of FeLV and its causative association with induction of leukemia, lymphoma, immune impairment, and hematosuppressive diseases led to its recognition as a unique naturally occurring gammaretrovirus.

Classification:

Originally oncogenic RNA viruses, such as FeLV and murine leukemia virus, were designated oncornaviruses; specifically, FeLV was classified as a mammalian C-type oncornavirus. Due to the characterization of virion RNA, these viruses are now assigned to various genera throughout the family Retroviridae. Thus, FeLV is now categorized within the subfamily Orthoretrovirinae genus *Gammaretrovirus*.

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Virion properties:

As is standard for retroviruses, FeLV contains a diploid single-stranded positive-sense RNA genome which is approximately 8.4 kb in length (11). Because each FeLV strand consists of only three genes (*gag*, *pol*, and *env*), it is considered a simple retroviral genome. The *gag* gene encodes the structural proteins p15 (matrix), p12, p27 (capsid), and p10 (nucleocapsid), the *pol* gene encodes the enzymes p13 (protease), p74 (reverse transcriptase), and p47 (endonuclease-integrase), and the env gene encodes the viral envelope components gp70 (surface glycoprotein) and p15E (transmembrane protein) (11, 24, 56, 71, 75). The internal structural proteins are produced in great excess and consequently, p27 is found not only in the cytoplasm of infected cells, but also on the cell surface and it is released from infected cells (24, 73, 76). The reverse transcriptase enzyme is an RNA-dependent DNA polymerase consequently, retroviruses replicate through a DNA intermediate: upon cell entry, a single FeLV RNA strand undergoes reverse transcription and the subsequent double strand DNA is then integrated into the host cell genome. During the process of reverse transcription, the 5'and 3' ends of the RNA strand are duplicated and added to each end of the resulting dsDNA. The three genes are bracketed by these redundant long terminal repeats (LTR) which contain three regulatory domains: 1) U3 is a region unique to the 3' end of the RNA strand; 2) R is a region repeated on both the 5' and 3' ends of the RNA strand; and 3) U5 is a region unique to the 5' end of the RNA strand. This integrated viral DNA is now referred to as provirus and will persist for the life of the cell. The provirus is the template for viral transcription and replication.

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Endogenous FeLV:

In addition to exogenous FeLV, genetically similar endogenous retrovirus sequences, enFeLV, are permanently integrated into felid genomes and are transmitted vertically through the germ line (79, 96). Approximately 6-12 copies of enFeLVs are randomly distributed throughout a haploid genome (55, 74). enFeLVs are polymorphic with major deletions in the *gag-pol* and *env* regions rendering these viruses non-infectious (78, 112, 113). However, subgenomic expression of endogenous sequences does occur (7, 67, 74, 113). Although enFeLVs preserve the LTR, the U3 region of the endogenous LTR displays considerable divergence from the exogenous U3 region (5, 8). It remains uncertain whether enFeLVs influence the course of exogenous FeLV infection (2, 4, 67, 88, 118).

Subgroups:

Cellular resistance to viral superinfection is mediated by pre-infecting viral interference with receptor binding of a challenge virus with identical viral envelope. Viral interference and virus neutralization assays enabled classification of FeLV into subgroups A, B, and C (107, 108). Subgroup A viruses are found in all naturally occurring isolates of FeLV, whereas both Subgroups B and C viruses are only found in conjunction with Subgroup A viruses (107, 108). Subgroup B occurs in ~50% of FeLV isolates and Subgroup C is found in ~1% of isolates (47). Originally, the three subgroups demonstrated different host ranges suggesting the presence of three cellular receptors (48, 109), although a recent report concluded that subgroup A viruses are not strictly ecotropic (72). Nonetheless, distinct receptor requirements for each subgroup have been

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identified. Subgroup A viruses use the thiamine transport protein 1 (feTHTR1) (68). Subgroup B viruses arise through recombination of FeLV-A with endogenous FeLV envelope sequences (115) resulting in a change in receptor specificity to a sodium-dependent phosphate symporter (Pit1 or Pit2 receptor) (3, 6, 117). The receptors feTHTR1, Pit1, and Pit2 are broadly expressed across many tissues (53, 68), consistent with the pattern of infection by FeLV-A and FeLV-B (64, 103). Subgroup C viruses arise through mutations in the envelope gene of FeLV-A (101) producing a change in receptor specificity to the heme exporter FLVCR1 (95, 116). The disease observed with FeLV-C infections (aplastic anemia) (64, 71) is compatible with a blockade or downregulation of FLVCR1. The identification of T-cell tropic cytopathic variants which induce fatal immunodeficiency may represent a fourth subgroup (FeLV-T) (81, 102). Several insertions, deletions, and substitutions within the FeLV-A envelope gene are responsible for altering the receptor usage to form these new FeLV-T variants (12, 70, 93, 102). A novel secreted extracellular protein (FeLV infectivity X-essory protein or FeLIX) functions in combination with Pit1 to permit FeLV-T infection (2). The T-cell tropism of FeLV-T is explained by the high expression of FeLIX in T cells (57).

Diagnosis:

The first diagnoses of FeLV were made by direct visualization of virions via electron microscopy (EM) (51, 52, 54, 99, 100). Since that time, significant advances have been made in sensitivity, specificity, and speed of FeLV diagnosis. Among the first of the FeLV diagnostic tests was the focus formation assay to detect infectious virus (VI). Because FeLV does not cause cytopathic effects, the clone 81 cell line was derived. This

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is a CCC cell line transformed by a sarcoma-positive, leukemia-negative Moloney murine sarcoma virus that reacts to high dilutions of replicating FeLV by the induction of lytic-type foci in the monolayer (10, 13). The copious production of p27 capsid antigen made possible the development of the direct immunofluorescent antibody (IFA) assay to detect p27 within leukocytes and platelets on blood films (28, 50). Because the detection of viremia and intracellular antigenemia was correlative (10, 28, 40, 46), it was assumed that the VI and IFA were both a measure of viral replication in bone marrow; consequently, use and results of these assays became interchangeable. Development of an antigen capture ELISA to detect extracellular p27 capsid antigen in plasma or sera using monoclonal antibodies directed against different epitopes of p27 provided a rapid and sensitive diagnostic assay for testing animals on site at low cost (61). Use of the VI, IFA, and ELISA assays in combination, however, led to recognition of cats with 'discordant' results. Extracellular (serum, plasma) viral antigen was detected in the absence of detectable antigen in circulating cells and/or infectious virus in plasma (29, 46, 62, 63). Because identification of FeLV infection has necessarily been based on assays that rely on viral replication and substantial viremia/antigenemia, the advent of molecular assays, which amplify the nucleic acid signal, offered an alternative. However, when conventional PCR detecting FeLV DNA was employed in attempt to better understand these discrepant results, it did not appear to have increased sensitivity or specificity compared with the antigen-capture ELISA as these assays had perfect agreement (32, 45, 69). More recently, modification of conventional PCR allowing quantitation of input DNA or RNA was developed. Quantitative real-time PCR (qPCR) is based on the 5'-3' exonuclease activity of the *Taq* DNA polymerase and the Förster

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resonance energy transfer (FRET) phenomenon that occurs when using a sequence-specific probe with a quencher dye and fluorescing reporter dye. The fluorescent signal of amplified DNA is monitored as it is generated, during the exponential phase of amplication well before the reaction reaches a plateau. Thus, with appropriate optimization and validation, qPCR offers increased sensitivity, specificity, speed, and also quantitative results. To verify amplification of exogenous FeLV but not enFeLV sequences, it is essential that a primer/probe set is designed within the U3 region of the LTR (5, 8). qPCR cannot distinguish between integrated provirus and unintegrated viral DNA; consequently, it is unknown whether the viral DNA detected by a qPCR assay represents intact, replication-competent provirus or replication-defective viral DNA sequences.

Transmission:

Most of the early observed cases of FeLV were clustered in unrelated cats living in multi-cat households, suggesting horizontal transmission (27). FeLV p27 antigen was demonstrated in lymphoid and hematopoietic tissues but its occurrence in salivary gland prompted further examination of epithelial tissues (25). FeLV virions were visualized in salivary tissue, urinary bladder, and urine of FeLV-infected cats (18, 50) and infectious virus was isolated from the saliva and intermittently from urine from such animals (17, 27). In addition, FeLV was experimentally transmitted via intranasal inoculation (35). Because levels of infectious FeLV excreted in saliva were found to be as high or higher than levels in blood (17), saliva was proposed as the most likely mode of transmission. FeLV cannot survive desiccation, (16) thus, transmission probably occurs via intimate

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contact (e.g. mutual grooming and biting) (26, 40). Recently, high levels of FeLV RNA have been demonstrated in saliva from viremic cats (19, 20). It appears that vertical transmission is an unlikely route of infection. Active FeLV infection during pregnancy most commonly results in early fetal death, or less commonly, congenital fetal infection (42). Pregnant queens with latent FeLV infection rarely transmit virus to their offspring but latent fetal infections and a single active fetal infection have been observed (42, 85). In addition, milk transmission from a intermittently antigenemic dam has also been identified (83).

Pathogenesis:

The pivotal work of Rojko *et al.* established the fundamental principles of FeLV pathogenesis (103). Following FeLV exposure, blood and tissues from 59 cats were serially examined for intracellular p27 antigen and six stages of FeLV replication were demonstrated. Initially, host cell contact is initiated in draining lymphoid tissues 2-14 days post-inoculation (dpi). A primary cell-associated viremia is then detected in circulating lymphocytes and monocytes which spread the virus to secondary sites 1-14 dpi. Amplification of virus then occurs in follicular lymphoid tissue (presumptive B cells regions) throughout the body (spleen, lymph nodes, GALT) 3-12 dpi. Next, extensive replication within hematopoietic cells of the bone marrow (principally megakaryocytes and developing platelets, and neutrophils) and intestinal crypt epithelium began between 7-21 dpi. The establishment of a secondary marrow-origin viremia ≥ 14-28 dpi is then detected in circulating neutrophils and platelets. Finally, widespread infection of

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mucosal and glandular epithelium (especially salivary glands, oropharynx, nasophaynx, and trachea) with viral shedding is detected ≥ 28-56 dpi.

FeLV:host relationships:

Available evidence suggests that the interplay between the host and virus within the first 4 weeks after FeLV exposure results in either: (a) persistent active (progressive) infection or (b) self-limiting (regressive) infection (41, 103).

Progressive Infection

Approximately 30% of cats exposed to FeLV have immune responses which fail to contain viral replication in the early phases of FeLV infection (26, 41, 103). Consequently, extensive viral replication occurs in lymph nodes, epithelia, and bone marrow precursor cells (41, 103). These animals develop persistent antigenemia and viremia, continuously excrete virus, and most die within a few years from FeLV-related disease (26, 41, 103). Leukocyte subsets from long-term persistently infected animals were analyzed for viral DNA and evidence of virus production. Using semi-quantitative PCR (92, 94), the highest viral DNA burden was demonstrated in B lymphocytes but provirus was also detected in CD4+ and CD8+ T lymphocytes whereas proviral loads were uniformly high in all leukocyte subsets when quantitative PCR was used (86). However, monocytes and granulocytes were found to have the most productive infections when analyzed for intracellular p27 capsid antigen (92, 94) and intracellular viral RNA levels (86), consistent with the marrow-origin viremia model.

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Regressive Infection

A substantial fraction of cats exposed to FeLV (~60%) have successful host immune responses which effectively contain virus during the initial stages of lymphoid tissue replication, before establishment of FeLV infection in the bone marrow (41, 103). Thus, such animals resist induction of persistent intracellular antigenemia and viremia, they do not shed virus, and they abrogate development of disease (41, 103). Such cats are resistant to subsequent FeLV challenge (41). Recently, viral DNA and RNA loads within leukocyte subsets were examined from non-antigenemic animals years after FeLV exposure (86). Viral DNA was carried primarily in CD4+ T cells and B cells. Moreover, viral DNA was not found in granulocytes, giving compelling evidence for a primary lymphocyte/monocyte-associated viremia with viral containment occurring before bone marrow involvement and the subsequent secondary granulocyte-associated viremia as originally posited by Rojko *et al.* (103).

Historically, this diametric paradigm has been based on assays for viral infectivity (VI) or viral p27 Gag antigen in blood cells by IFA. However, subsequent widespread use of the p27 ELISA, in combination with the IFA and VI assays, prompted the identification of cats with 'discordant' results (29, 46, 62, 63). The concomitant demonstration of latent and atypical infections gave the first intimation that more nuanced host:virus relationships, beyond the polar infection concept, were in play and/or the available assays were simply limited by sensitivity.

Latent infection

Investigators demonstrated that 30-60% of non-antigenemic/non-viremic cats that experienced regressive infection retained non-productive/latent FeLV infections in bone

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marrow (66, 85). These non-productive infections were reactivated by *in vivo* or *in vitro* administration of corticosteroids or by cultivation of explanted bone marrow cells (66, 85, 91, 104). Presumably, such animals do not contain virus before establishment of FeLV infection in the bone marrow and thus, the FeLV genome is present but infectious virus particles are not produced (36, 105). Although horizontal transmission through contact with latently-infected cats has not been observed (66), vertical transmission has infrequently been documented in latently-infected dams to their offspring resulting in latent fetal infections and a single active fetal infection (42, 85). In addition, FeLV-related disease rarely develops and the incidence of latency decreases over time (82, 85). Several attempts to amplify viral DNA sequences in circulating and/or bone marrow cells from cats with suspected latent infections have been unsuccessful using conventional PCR (32, 45, 69).

Atypical infection

Rarely, incomplete viral containment occurs and productively infected cells supposedly remain in sequestered sites (36, 105). Although such animals may have extracellular (serum, plasma) antigenemia, the absence of detectable antigen in circulating cells and/or infectious virus in plasma lead some to believe the virus was harbored in some reservoir tissue. In one situation, infectious virus was only detected in the urine of an animal with extracellular antigenemia (63). In another case, an intermittently antigenemic but non-viremic animal transmitted FeLV via milk (83). Another animal had sufficient virus in plasma to transmit FeLV to others but was non-antigenemic and non-viremic (31). These atypical FeLV:host relationships were most likely the result of limited sensitivity of available assays.

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Immunity:

There are several determinants which influence the FeLV:host relationship. A host age-related resistance has been demonstrated whereby 100% of neonatal kittens are susceptible to FeLV, 85% of 2 week- to 2 month-old cats, and only 15% of cats older than 4 months are susceptible to FeLV (39). This natural resistance has been linked to macrophage maturity (43). Not surprisingly, passive immunity has conferred protection against FeLV infection (44, 49, 110). These virus neutralizing (VN) antibodies are directed against the surface glycoprotein gp70 at a site distinct from the receptor binding site (97, 106). Although VN antibodies have been demonstrated in non-viremic animals, responses have been modest and less than consistent (9, 21, 24, 26, 39, 66, 83, 106). Moreover, FeLV vaccine studies have observed that resistance to infection was attained despite the absence of a detectable VN antibody response (23, 30, 84). This lack of association between humoral responses and effective viral containment suggests a role for protective cell-mediated or other innate immune responses. Indeed, Flynn *et al.* (14, 15) have demonstrated an association between moderate levels of FeLV-specific cytotoxic T lymphocyte activity in cats that resisted infection. However, the determinants of effective FeLV immunity remain far from understood.

FeLV-related diseases:

Due to the age-related variation, young cats may die from acute immunosuppression shortly after the onset of viremia (1, 35, 65). However, the typical time until development to FeLV-related diseases in most infections is months to years (26). The pathogenic effects of FeLV are paradoxical, causing both cytoproliferative and

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cytosuppressive disease (36, 41). The cytoproliferative diseases include lymphoma, leukemia, fibrosarcoma, and myeloproliferative disorders, with lymphoma occurring most frequently (24, 26, 36, 41, 52, 100, 111). Despite its name, FeLV more commonly causes cytosuppressive diseases such as immunodeficiency and myelosuppression (1, 24, 34, 36, 41, 64, 77, 80, 87). In addition, FeLV is responsible for causing enteropathies, fetal resorption/infertility syndrome, and neurologic syndromes (36, 41, 42, 98).

Vaccination:

That effective host containment of FeLV infection can occur prompted research leading to development of the first commercial vaccine for a naturally occurring retroviral infection (59). An abundance of vaccine trials have evaluated commercial vaccine efficacy, reviewed by Loar (60) and Sparkes (114). Because these studies have used varying challenge viruses, dosages, routes, and ages of vaccinates, it is difficult to make meaningful comparisons. Nonetheless, it appears the whole inactivated virus (WIV) vaccines, Fel-O-Vax Lv-K® (Fort Dodge Animal Health, Overland Park, KS) and FEVAXYN FeLV® (Schering-Plough Animal Health Corporation, Summit, NJ), show the most consistent protection against FeLV challenge by comparison of preventable fractions. The preventable fraction (PF) is used to express vaccine efficacy due to the inherent resistance of some unvaccinated cats to development of persistent antigenemia after FeLV challenge (89). Although the method to determine persistent antigenemia/viremia varies from study to study, the PF is calculated as

$$\frac{\text{Incidence of Persistent Antigenemia in Controls} - \text{Incidence of Persistent Antigenemia in Vaccinates}}{\text{Incidence of Persistent Antigenemia in Controls}}$$

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The PF for Fel-O-Vax Lv-K® was previously reported as 86 – 100% (37, 38, 58) and the PF for FEVAXYN FeLV® was 90 – 100% (33, 84). In comparison, the adjuvanted, inactivated mixed subunit vaccine LEUKOCELL 2® (Pfizer Animal Health, New York, NY) was reported to have a PF of 35 – 88% (22, 23, 58, 90, 119).

Dissertation research:

A substantial fraction of cats exposed to FeLV effectively contain virus and resist induction of persistent antigenemia and viremia. Little is known, however, about the state of the viral genome in these animals. To further explore FeLV:host relationships using more sensitive nucleic acid-based detection methods, we developed a quantitative real-time PCR (qPCR) assay to detect FeLV DNA in blood cells and tissues. We examined FeLV-vaccinated and unvaccinated cats for viral DNA sequences in circulating cells during the early (weeks post-challenge) phase of FeLV infection and both circulating cells and tissue during the late (years post-challenge) phases of FeLV infection. We hypothesized self-limiting infections were non-productive and viral DNA was not detectable in circulating cells but latent viral DNA was retained in tissue. It was unknown, however, whether the FeLV DNA we detected by our qPCR assay represented intact, replication-competent provirus or viral DNA that was either not integrated into the host cell genome or was otherwise replication-defective. We hypothesized the FeLV infection was indeed productive and the absence of antigenemia was due to limits in sensitivity of the antigen detection by ELISA. To determine whether the viral DNA detected was transcriptionally active, we developed a reverse transcriptase qPCR assay to quantitate extracellular FeLV RNA in plasma and compared these results with those for

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viral DNA, p27 capsid antigen capture ELISA, and viral infectivity assays in FeLV-vaccinated and unvaccinated cats challenged with FeLV-A/61E during the early phase of FeLV infection. In addition we assessed the level of virus neutralizing (VN) antibody produced post-vaccination and again post-challenge as a means to identify immune correlates of protection. In conclusion, we have used contemporary methodologies increasing the sensitivity of FeLV detection to study innate and vaccine-primed FeLV resistance because the mechanisms responsible for effective vs. ineffective viral containment are central to immunoprevention and therapies of retroviral infections.

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CHAPTER 1*

RE-EXAMINATION OF FELINE LEUKEMIA VIRUS:HOST RELATIONSHIPS USING REAL-TIME PCR

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ABSTRACT

The mechanisms responsible for effective vs. ineffective viral containment are central to immunoprevention and therapies of retroviral infections. Feline leukemia virus (FeLV) infection is unique as a naturally occurring, diametric example of effective vs. ineffective retroviral containment by the host. We developed a sensitive quantitative real-time DNA PCR assay specific for exogenous FeLV to further explore the FeLV:host relationship. By assaying p27 capsid antigen in blood and FeLV DNA in blood and tissues of successfully vaccinated, unsuccessfully vaccinated, and unvaccinated pathogen-free cats, we defined four statistically separable classes of FeLV infection, provisionally designated as: abortive, regressive, latent, and progressive. These host:virus relationships were established by 8 weeks post-challenge and could be

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maintained for years. Real-time PCR methods offer promise in gaining deeper insight into the mechanisms of FeLV infection and immunity.

INTRODUCTION

Feline leukemia virus (FeLV) is a naturally occurring, contagiously transmitted, gammaretrovirus of cats (16, 21, 29, 30, 49). Its pathogenic effects are paradoxical, causing both cytoproliferative (e.g. lymphoma or myeloproliferative disorder) and cytosuppressive (e.g. immunodeficiency or myelosuppression) disease (2, 5, 6, 20, 29, 30, 36, 46, 49). While many FeLV-exposed cats (estimated at ~30%) develop progressive infection and FeLV-related disease, at least twice as many (estimated at ~60%) develop regressive infection marked by an effective and durable immune response which contains and possibly extinguishes viral replication, thereby abrogating development of disease (15, 22, 27, 50). That effective host containment of FeLV infection can occur prompted research leading to development of the first vaccine for a naturally occurring retroviral infection (26, 34, 53).

Available evidence suggests that the interplay between the host and virus within the first 4 weeks after FeLV exposure results in either: (a) failure of host immune response to contain viral replication in lymph nodes, epithelia, and bone marrow precursor cells or (b) successful host immune response resulting in curtailment of viral replication (22, 27, 50). Cats with progressive infection develop persistent antigenemia as detected by p27 capsid antigen capture in blood and have neither virus neutralizing antibodies (VN Ab) nor high levels of FeLV-specific cytotoxic lymphocytes (CTLs) (10,

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11, 22). By contrast, cats with regressive infection do not develop persistent antigenemia but do produce VN Ab and a detectable CTL response (10, 11, 22). Because identification of FeLV infection has necessarily been based on assays that rely on viral replication and substantial viremia/antigenemia, it is unclear whether regressors retain latent (non-productive) infection or instead may eliminate all cells bearing integrated FeLV provirus. Several laboratories have shown that it is possible to reactivate FeLV from some cats with regressive infection (37, 48, 51). Despite this, attempts by other laboratories to amplify viral DNA sequences in circulating and/or bone marrow cells from cats with suspected latent infections have been unsuccessful using conventional PCR (18, 28, 38). Similar to FeLV regressors, protected vaccinates do not develop persistent antigenemia. To the authors' knowledge, however, studies assessing vaccinates for potential latent infections using PCR have not been performed (53).

Recent studies employing quantitative real-time PCR in experimental FeLV infections have shown that the early circulating proviral burden influences the course of infection and that real-time PCR detected provirus in circulating cells from cats with undetectable or transient antigenemia (10, 19). To explore further the FeLV:host relationship and assess the presence of latent viral DNA in circulation and tissue, we developed a quantitative real-time PCR assay and examined the early (weeks post-challenge) and late (years post-challenge) phases of experimental FeLV infection in both unvaccinated animals and those primed by vaccination. Here we examine proviral and p27 levels in FeLV-A/61E-challenged cats given effective, ineffective, or no FeLV vaccine. Based on the results of these studies we suggest four categories within the

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spectrum of FeLV infection, which we have provisionally designated as abortive, regressive, latent, and progressive.

MATERIALS AND METHODS

Study Design

Samples were utilized from two previous vaccine experiments. Experiment 1 consisted of 5 groups: group 1 received Vaccine A, groups 2, 3, and 4 all received Vaccine B but each by different routes of administration, and group 5 served as the Control as these cats did not receive any vaccination. Using repeated-measures ANOVA, no statistically significant differences were detected between the 3 groups which received Vaccine B by different routes of administration ($p = 0.47$, power = 0.15). Consequently, results from the 3 groups which received Vaccine B were combined. Experiment 2 consisted of 2 groups: group 1 received Vaccine A and group 2 served as the Control as these cats did not receive any vaccination. Again, no statistically significant differences were detected between the Vaccine A groups from Experiment 1 and 2 ($p = 0.16$ power = 0.27) or between the Control groups from Experiment 1 and 2 ($p = 0.53$, power = 0.09). Thus, results from the Vaccine A groups from Experiment 1 and Experiment 2 were combined and results from the Control groups from Experiment 1 and Experiment 2 were combined. In summary, this study presents the results from a combined total of 3 groups: Vaccine A, Vaccine B, and Control (Table 1.1).

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Table 1.1. Summary of study design.

Group	# of cats	Prime	Boost	Weeks of age at challenge	# of cats necropsied	Weeks post-challenge at necropsy
Vaccine A*	5[1]	SQ[3]	SQ	22-23	5	90
	5[2]	SQ	SQ	34-36	--	--
Vaccine B**	5[1]	IN[4]	IN	22-23	1	153
	5[1]	SQ	IN	22-23	2	153
	5[1]	SQ	--	22-23	1	153
Control***	5[1]	--	--	22-23	2	153
	5[2]	--	--	34-36	2	177

* = Fel-O-Vax Lv-K® (Fort Dodge Animal Health)
** = Experimental whole inactivated FeLV-Sarma-A with MPL adjuvant
*** = No vaccine
[1] Experiment 1
[2] Experiment 2
[3] Subcutaneous administration of vaccine
[4] Intranasal administration of vaccine

Experimental Animals

Thirty-five specific-pathogen-free (SPF) cats were obtained from Cedar River Laboratories (Mason City, IA) and randomly divided into 7 groups, each group consisting of 5 cats (Table 1.1). Each group was individually housed at Laboratory Animal Resources at Colorado State University (Fort Collins, CO) in accordance with the University Animal Care and Use Committee regulations. Vaccination, virus challenge, and all sample collections were performed on cats anesthetized with a subcutaneous administration of ketamine hydrochloride (22mg/kg) and acepromazine maleate (0.1mg/kg).

Vaccination

Ten cats were administered Vaccine A, the commercial FeLV vaccine Fel-O-Vax Lv-K® (Fort Dodge Animal Health, Overland Park, KS) (23, 24), according to the

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manufacturer's specifications (Table 1.1). Five cats received the subcutaneous priming vaccination at 15-16 weeks of age and a subcutaneous boosting vaccination at 19-20 weeks of age. The other five cats received the prime at 25-27 weeks of age and the boost at 31-33 weeks of age. Fifteen cats were administered Vaccine B, an experimental whole inactivated FeLV-Sarma-A with monophosphoryl lipid A adjuvant (MPL®) (Corixa Corp., Seattle, WA), by different routes of administration. All 15 cats received the priming vaccination at 15-16 weeks of age and a boosting vaccination at 19-20 weeks of age. Five cats received an intranasal prime and boost, 5 cats received a subcutaneous prime and an intranasal boost, and 5 cats received a subcutaneous prime and no boost. Ten cats did not receive any vaccinations and served as the Controls.

Virus challenge

All cats were challenged oronasally with 1 mL of 10^4 TCID/mL FeLV-A/61E via dropwise instillation of 0.25mL in each nostril and 0.5mL in the mouth. This subgroup A virus strain is the highly replication competent, non-acutely pathogenic component of the FeLV-FAIDS complex (9, 25, 39, 42). The cell-free infectious virus inoculum was prepared as supernatant from Crandell feline kidney (CrFK) cell cultures and determined to be equivalent to 1 CID_{100} (100% cat infective dose). The vaccinated cats were challenged three weeks after receiving their boosting immunization; either 22-23 or 34-36 weeks of age (Table 1.1). Five control cats were challenged at 22-23 weeks of age and the other five at 34-36 weeks of age. All cats were observed daily for signs of illness after virus inoculation.

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Sample collection and processing

Blood samples were collected at challenge and every 2 weeks thereafter through 8 weeks post-challenge (PC). Sera were stored at -20°C until analysis for FeLV p27 capsid antigen by capture ELISA. Peripheral blood mononuclear cells (PBMC) were isolated from blood by Ficoll-Hypaque (Histopaque®-1077; Sigma Diagnostics, St. Louis, MO) density gradient centrifugation, separated into 1×10^6 PBMC/mL aliquots, and stored at –80°C until analysis by FeLV quantitative real-time PCR. DNA was extracted from PBMC using a QIAamp® DNA blood mini kit (Qiagen, Inc., Valencia, CA), eluted in 100μL of elution buffer, and DNA concentrations determined spectrophotometrically.

Thirteen of the 35 cats were available for necropsy after long-term survival periods. Five cats from the Vaccine A group were necropsied at 90 weeks PC, 4 cats from the Vaccine B group and 2 from the unvaccinated Control group were necropsied at 153 weeks PC, and 2 cats from the unvaccinated Control group were necropsied at 177 weeks PC (Table 1.1). Blood was collected and processed as above. The thymus, tonsil, retropharyngeal lymph nodes, bone marrow (BM), spleen (SP), and mesenteric lymph nodes (MLN) were collected from the 5 Vaccine A cats. BM, SP, and MLN were collected from the 4 Vaccine B and 4 unvaccinated control cats. Tissues were stored at –80°C until analysis by FeLV quantitative real-time PCR. DNA was extracted and RNA digested from tissues using a QIAamp® DNA mini kit and RNase A (Qiagen, Inc.), respectively, eluted in 100μL of elution buffer, and DNA concentrations determined spectrophotometrically.

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Detection of circulating p27 capsid antigen by capture ELISA

FeLV p27 capsid antigen was detected in serum by capture ELISA using the monoclonal antibodies (mAbs) anti-p27 A2 and G3 (35) (kindly provided by Niels C. Pedersen; University of California, Davis, CA) as previously described (55) with minor adaptations. Briefly, 0.5μg/well of the primary mAb, G3, was used to coat a 96-well plate, 50μL of control or sample sera was added in duplicate to plate wells, and 50μL of the secondary horseradish peroxidase-conjugated mAb, A2 at 1:250, was added and incubated for 45 minutes. The plates were then rinsed and 100μL/well TMB peroxidase substrate:peroxidase solution B (H_2O_2) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added for color development. After a 15-minute incubation, reactions were stopped with 50μL/well 2N H_2SO_4 and optical density measurements were taken at 450 nm. Background readings, using FeLV-naïve SPF cat serum, were subtracted from each well. Sample well reactions were considered positive if an absorbance value of 10% or more of the positive control (persistent antigenemic FeLV-infected cat serum) was obtained.

Detection and quantification of circulating and tissue FeLV viral DNA by quantitative real-time PCR

Using Primer Express® software (Applied Biosystems, Foster City, CA), we designed a primer/probe set within the U3 region of the FeLV-A/61E long terminal repeat (LTR) (GenBank accession number M18247) (9), thereby amplifying the exogenous but not endogenous FeLV sequences (3, 4). The forward, 5' AGTTCGACCTTCCGCCTCAT 3' (20 bases; nt 241-260), and reverse, 5'

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AGAAAGCGCGCGTACAGAAG 3' (20 bases; nt 308-289), primer sequences amplified a 68bp fragment. The corresponding probe sequence, 5' TAAACTAACCAATCCCCATGCCTCTCGC 3' (28 bases; nt 262-289), was labeled with the reporter dye, FAM (6-carboxyfluorescein), at the 5' end and the quencher dye, TAMRA (6-carboxytetramethyl-rhodamine; Applied Biosystems) or BHQ-1 (Black Hole Quencher-1; Biosource International, Inc., Camarillo, CA), at the 3' end. Both probes were blocked at the 3' end to prevent extension. The two probes produced similar results.

The 25μL reaction consisted of 400nM of each primer, 80nM of fluorogenic probe, 12.5μL of TaqMan® Universal PCR Master Mix (Applied Biosystems), 3.5μL of PCR-grade H_2O, and 5μL of sample or plasmid standard DNA. The master mix was supplied at a 2X concentration and contained AmpliTaq® Gold DNA Polymerase, AmpErase® uracil N-glycosylase (UNG), dNTPs with dUTP, and optimized buffer components. Reactions were performed in triplicate using an iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Every reaction plate contained a template control (no DNA, PCR-grade H_2O only) and a negative control (FeLV-naïve, SPF cat DNA). Thermal cycling conditions were 2 minutes at 50°C to allow enzymatic activity of UNG, 10 minutes at 95°C to reduce UNG activity, to activate AmpliTaq® Gold DNA Polymerase, and to denature the template DNA, followed by 40 cycles of 15 seconds at 95°C for denaturation and 60 seconds at 60°C for annealing/extension.

The plasmid p61E-FeLV, an *Eco*RI fragment containing the full-length FeLV-A/61E provirus subcloned into pUC18 (9, 42), was used as the standard for PCR

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quantification. The plasmid was provided as ampicillin-resistant transformed *E. coli* JM109 cells through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. James Mullins. The transformed *E. coli* cells were grown on LB media containing 50μg/mL ampicillin. Plasmid DNA was isolated from the bacterial cells using the QIAfilter™ plasmid midi kit (Qiagen, Inc.), linearized with *Eco*RI, and the full-length FeLV-A/61E fragment was confirmed by agarose gel electrophoresis with ethidium bromide staining. The linearized plasmid standard copy number was calculated from optical density measurements at 260 nm. A 10-fold dilution series of the plasmid standard template DNA was made in 1X TE buffer with 40ng/μL salmon testes DNA (Sigma Chemical Co., St. Louis, MO) as a carrier. Quantification of the sample amplicon was achieved by comparing the threshold cycle (C_T) value of the sample DNA with the standard curve of the co-amplified standard template DNA. Cell numbers were calculated by assuming 6pg DNA/cell.

Analytical specificity and sensitivity of FeLV quantitative real-time PCR

Following agarose gel electrophoresis confirmation with GelStar® (BioWhittaker Molecular Applications, Rockland, ME) staining, the 68bp PCR products from two separate reactions were sequenced to verify analytical specificity. The TOPO TA Cloning® Kit (with pCR®2.1-TOPO® vector) (Invitrogen Corp., Carlsbad, CA) was used for cloning the amplicons prior to sequencing. Briefly, the PCR products were directly ligated into the linearized pCR®2.1-TOPO® vector (Invitrogen Corp.), the constructs were transformed into One Shot® TOP 10 chemically competent *E. coli* cells (Invitrogen Corp.), and the cells grown on LB media with 50μg/mL ampicillin using

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blue/white screening. Plasmid DNA was isolated from the bacterial cells using the QIAfilter™ plasmid midi kit (Qiagen, Inc.), linearized with *Eco*RI, and the plasmid insert confirmed by agarose gel electrophoresis with GelStar® (BioWhittaker Molecular Applications) staining. Two cloned inserts were sequenced by Davis Sequencing LLC, Davis, CA. The sequences of the PCR products were then aligned with FeLV-A/61E using BLAST® (1, 54) on the National Center for Biotechnology Information website.

End-point dilution experiments of the p61E-FeLV plasmid standard were performed to assess analytical sensitivity. A dilution series of 500, 100, 50, 10, 5, 1, 0.5, and 0.1 copies of the plasmid standard, each in triplicate, was tested.

Amplification efficiency and reproducibility of FeLV quantitative real-time PCR

To assess amplification efficiencies, serial dilutions (1:10, 1:100, 1:1000, and 1:10000) of PBMC DNA from an experimentally FeLV-A/61E-infected cat and of the p61E-FeLV plasmid standard were amplified in triplicate and the difference in the slopes (Δs) of the regression lines (C_T vs. dilution) was evaluated.

To assess assay reproducibility, dilutions of the p61E-FeLV plasmid standard (50000, 5000, and 500 copies) and of DNA from an experimentally FeLV-A/61E-infected cat (100%, 1:100, and 1:1000) were evaluated for within-run and between-run precision. Each dilution was run 10 times within the same reaction plate and between 10 different reaction plates to test the within-run and between-run precision, respectively. The coefficients of variations (CV) of the threshold cycles (C_T) were calculated: CV (C_T).

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Data analysis

Statistically significant differences in p27 and viral DNA levels (log transformed) between the experimental groups and between the FeLV:host categories were determined using repeated-measure analysis of variance (ANOVA) with the Tukey-Kramer post-hoc test. The kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance, between the p27 capture ELISA and the real-time PCR assay. Pearson correlation coefficients were determined to assess the linear relationship between circulating p27 levels vs. PBMC viral DNA levels and between circulating vs. tissue viral DNA levels. After a Fisher's r to z transformation, p values were obtained. A statistically significant difference between groups was considered to have occurred when a p value was <0.05. Repeated-measures ANOVA, the Tukey-Kramer post-hoc test, and the Pearson correlation coefficient were performed using StatView® version 5.0.1 for Macintosh, copyright 1999 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Validation of FeLV quantitative real-time PCR

Specificity

The analytical specificity of the FeLV quantitative real-time PCR assay was confirmed by sequencing two amplicons after agarose gel confirmation. Using BLAST® (1, 54), the amplicon sequences were shown to be identical to that of FeLV-A/61E.

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Peripheral blood mononuclear cells (PBMC) and lymphoid tissues from FeLV-naïve, specific-pathogen-free (SPF) cats were consistently negative for FeLV DNA (49/49 samples from 18 cats), thus, endogenous FeLV sequences were not amplified. Consequently, diagnostic specificity was 100% in FeLV-A/61E-infected animals.

Sensitivity

The analytical sensitivity of the FeLV real-time PCR assay was assessed in end-point dilution experiments. These studies consistently detected 5 copies of the p61E-FeLV plasmid standard (Fig. 1.1). The amplification signals of template control (no DNA, PCR-grade H_2O only), negative control (FeLV-naïve, SPF cat DNA), and samples containing 0.5 copy of the plasmid standard never crossed threshold. All FeLV-A/61E-infected cats that tested positive for p27 capsid antigen also were positive by real-time PCR (76/76 samples from 23 cats) (Table 1.2). Thus, diagnostic sensitivity in the animals studied was 100%.

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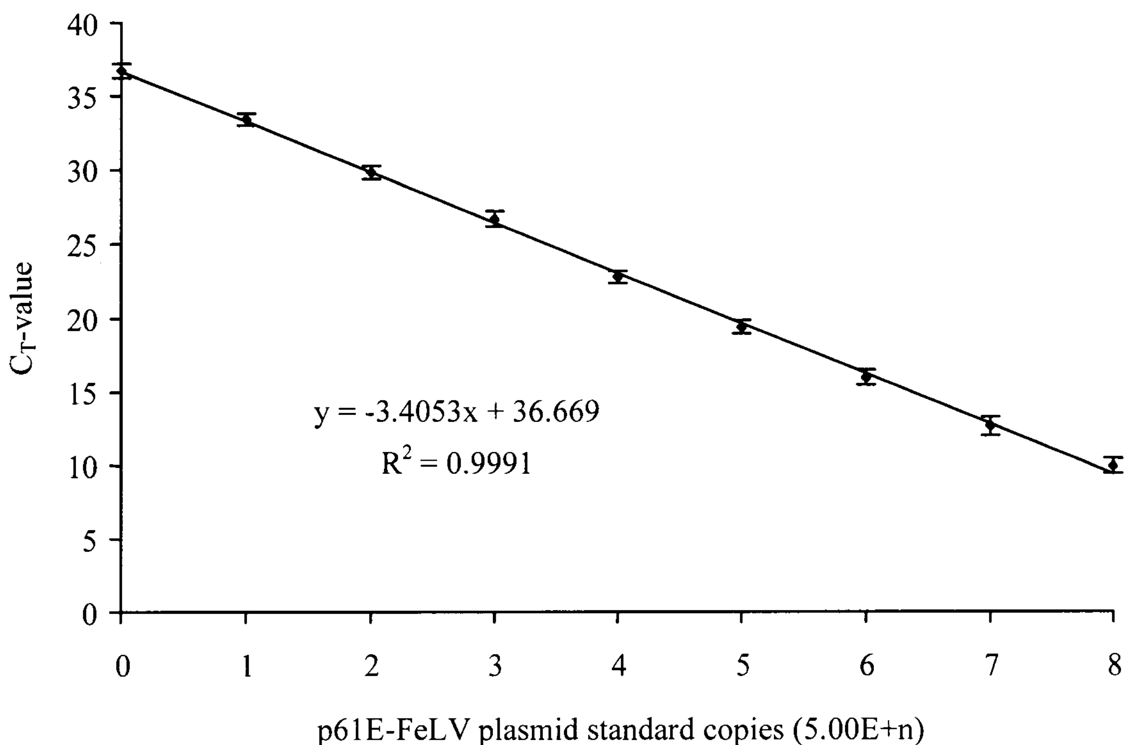

Fig. 1.1. Linearity and sensitivity of quantitative real-time FeLV DNA PCR. Standard curve of the p61E-FeLV plasmid is linear. Five copies of the plasmid are consistently detected. Amplification of ten-fold serial dilutions between 5 X 10^8 copies to 5 X10^0 copies of the plasmid demonstrated linearity over eight orders of magnitude, generated a standard curve correlation coefficient of 0.999, and produced an amplification efficiency of 96.6%. Mean ± SD for 18 independent experiments are plotted.

Table 1.2. Real-time DNA PCR vs. p27 capsid capture ELISA for FeLV detection.

		Real-time PCR		Total
		(+)	(-)	
p27 ELISA	(+)	76	0	76
	(-)	24	23	47
Total		100	23	123

Kappa value = 0.53 (fair agreement)

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Linearity

The linear range of the plasmid standard curve was evaluated. Amplification of ten-fold serial dilutions starting at 5 X 10^8 copies and ending at 5 X10^0 copies of the p61E-FeLV plasmid standard from 18 independent experiments demonstrated linearity over 8 orders of magnitude, generated a standard curve correlation coefficient of 0.999, and produced an amplification efficiency (31) of 96.6% (Fig. 1.1).

Amplification efficiency

The amplification efficiencies of FeLV-A/61E-infected cat DNA and the p61E-FeLV plasmid standard were compared to validate quantification using the plasmid standard. Equivalent amplification efficiencies are indicated by regression line slopes (s) with less than 0.1 difference (Δs) (13). The observed amplification efficiencies of the target DNA (s=3.32, R^2=0.997) vs. the plasmid standard (s=3.30, R^2=0.999) had a Δs=0.02 (Fig. 1.2). Thus, quantification using the plasmid standard was expected to be valid.

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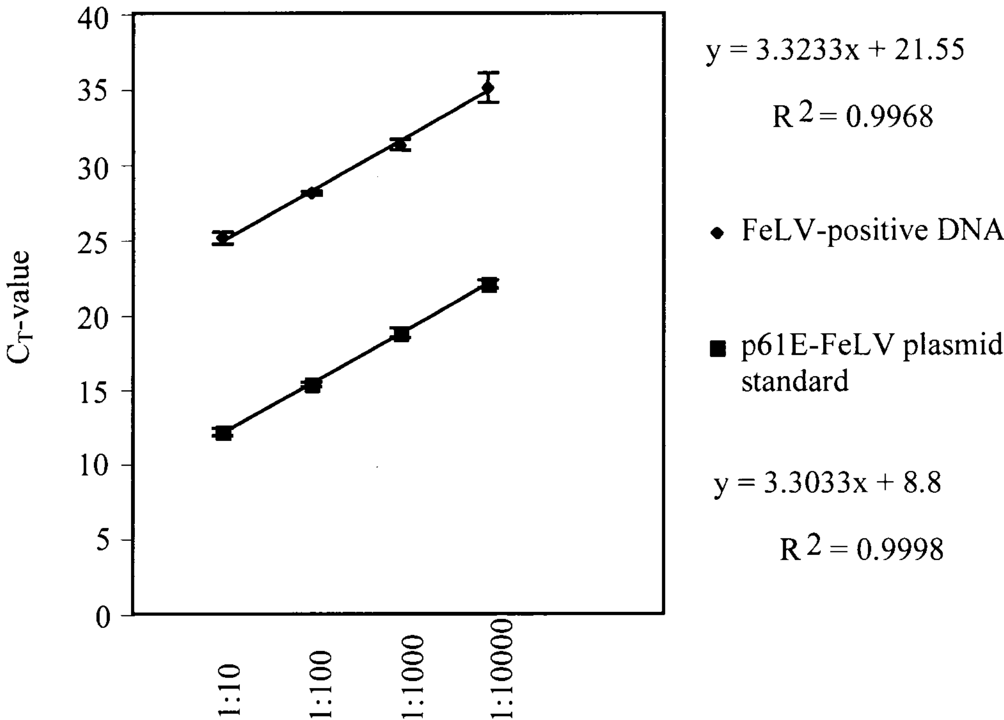

Fig. 1.2. Amplification efficiency comparison to validate real-time PCR quantification. Serial dilutions (1:10, 1:100, 1:1000, and 1:10000) of PBMC DNA from an FeLV-A/61E-infected cat and of the p61E-FeLV plasmid standard were amplified. Amplification efficiencies of the FeLV-positive DNA and the FeLV plasmid standard were approximately equal ($\Delta s=0.02$) demonstrating that quantification using the plasmid standard is expected to be valid. Mean ± SD are plotted.

Reproducibility

The within-run and between-run precision of the FeLV real-time PCR assay was evaluated. Several dilutions of the p61E-FeLV plasmid standard and of FeLV-A/61E-infected cat DNA were amplified 10 times within the same reaction plate and between 10 different reaction plates. The threshold cycle coefficients of variation, CV(C_T), for the within-run precision was 0.31 to 1.11% and the CV(C_T) for the between-run precision was 0.56 to 1.16% (Table 1.3). Thus, the assay was considered highly reproducible.

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Table 1.3. Real-time DNA PCR coefficients of variations (%) of within-run and between-run precision.

	Standard DNA (copies)			FeLV-positive DNA (dilution)		
	5×10^2	5×10^3	5×10^4	neat	1:100	1:1000
CV (C_T) within-run	0.45	1.11	0.31	0.83	0.33	0.83
CV(C_T) between-run	0.59	0.66	0.70	0.56	0.68	1.16

Early circulating p27 and viral DNA levels in FeLV-challenged animals

Sera and PBMC collected pre-challenge and every 2 weeks thereafter through 8 weeks post-challenge (PC) were analyzed for FeLV p27 capsid antigen via capture ELISA and for FeLV U3 LTR DNA via quantitative real-time PCR. None of the cats had detectable antigen or viral DNA pre-challenge.

Animals receiving Vaccine A

FeLV p27 was never detected in 9 of the 10 cats (90%) which received Vaccine A (Fig. 1.3 A). Of these 9 protected vaccinates, 4 never had detectable viral DNA, 2 developed transient low provirus loads (median: 130 copies/10^6 PBMC; range: 0 to 1,723 copies/10^6 PBMC) which gave way to undetectable levels (1 cat by 6 weeks and 1 cat by 8 weeks), and 3 had persistent low viral DNA levels (median: 225 copies/10^6 PBMC; range: 0 to 7,744 copies/10^6 PBMC) (Fig. 1.3 B). In the one persistently antigenemic failed vaccinate, a persistent high proviral burden was present PC (median: 477,999 copies/10^6 PBMC; range: 17,140 to 578,572 copies/10^6 PBMC).

Animals receiving Vaccine B

Thirteen of the 15 cats (86%) given Vaccine B developed persistent antigenemia and persistent high proviral burdens PC (median: 259,013 copies/10^6 PBMC; range:

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7,330 to 2,224,869 copies/10^6 PBMC). p27 was never detected in the remaining 2 vaccinates. In one of these latter animals, viral DNA also was never detected whereas in the second animal, persistent low proviral load (median: 18,790 copies/10^6 PBMC; range: 6,079 to 30,854 copies/10^6 PBMC) was present.

Unvaccinated Controls

Seven of the 10 unvaccinated control cats (70%) developed persistent antigenemia and high proviral burdens PC (median: 265,572 copies/10^6 PBMC; range: 11,942 to 1,508,006 copies/10^6 PBMC). The remaining 3 animals experienced transient antigenemia between 2 and 6 weeks PC, after which p27 was no longer detectable (1 cat by 4 weeks and 2 cats by 6 weeks). These latter cats retained persistent moderate proviral burdens (median: 40,969 copies/10^6 PBMC; range: 860 to 328,249 copies/10^6 PBMC).

Using repeated measures-ANOVA and the Tukey-Kramer post-hoc test, statistically significant differences ($p<0.01$) in p27 and viral DNA levels were present between Vaccine A vs. Vaccine B and between Vaccine A vs. unvaccinated Controls. Results for Vaccine B were not statistically different from the unvaccinated Controls.

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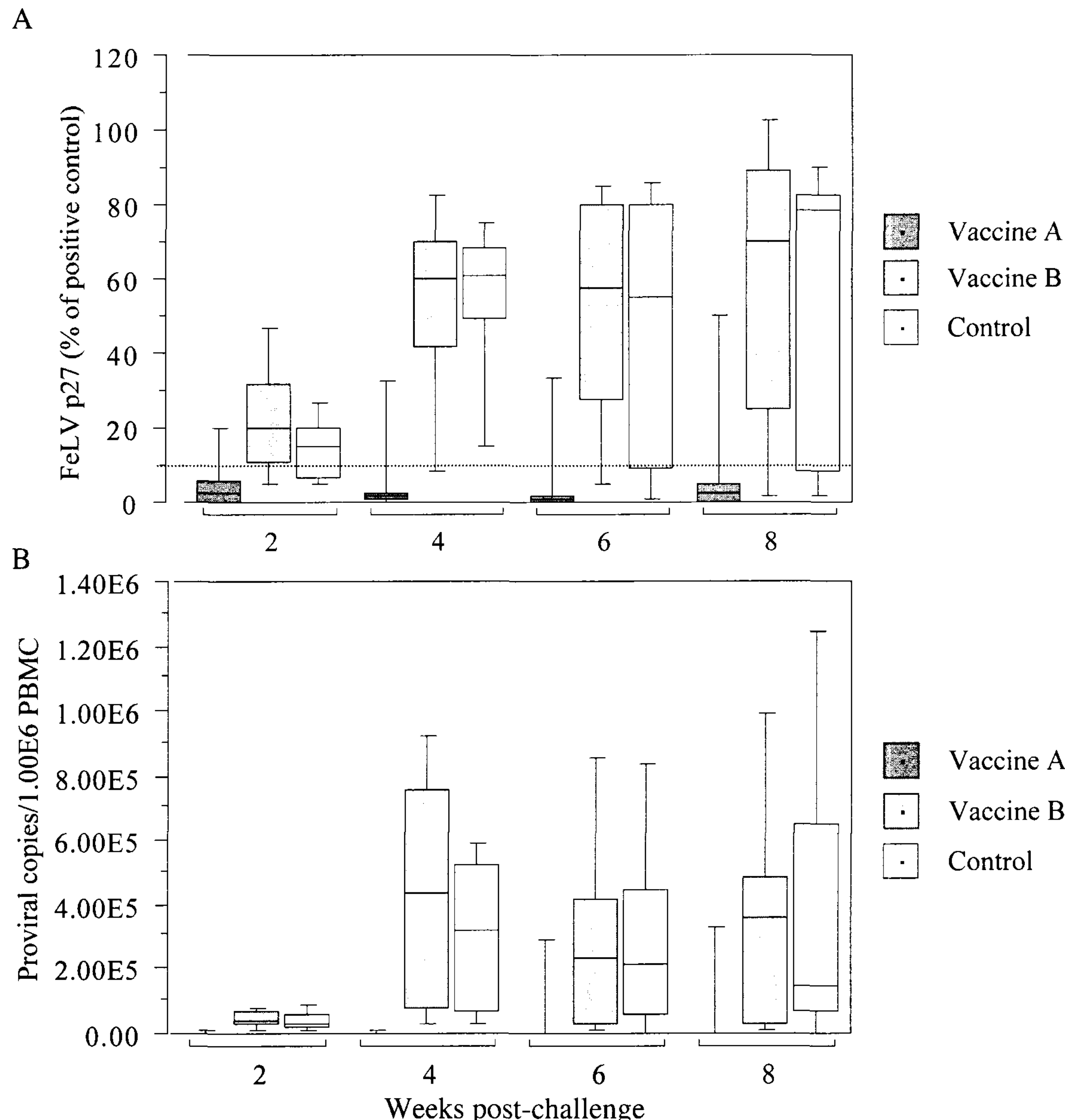

Fig. 1.3. Vaccine A (Fort Dodge Fel-O-Vax Lv-K®) protected cats against FeLV challenge: nine of 10 vaccinated cats did not develop detectable antigenemia and had low to undetectable proviral burden. Sera and PBMC collected at challenge and every 2 weeks thereafter through 8 weeks PC were analyzed for FeLV p27 capsid antigen via capture ELISA (A) and for FeLV DNA via quantitative real-time PCR (B). Only 1 of 10 Vaccine A cats developed persistent antigenemia with persistent high proviral burden. By contrast, 13 of 15 Vaccine B cats and 7 of 10 unvaccinated control cats developed persistent antigenemia and high proviral burdens. Statistically significant differences ($p<0.01$) for both p27 and viral DNA levels were detected between Vaccine A vs. Vaccine B and Vaccine A vs. unvaccinated Controls. Results for Vaccine B were not statistically different from the unvaccinated Controls. Graphed boxplots show the 10th, 25th, 50th (median), 75th, and 90th percentiles of a variable. Values above the 90th and below the 10th percentile are not shown. (A) The dotted line represents the threshold for positive results ($\geq$10% of the positive control).

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Preventable fraction

The preventable fraction (PF) is used to express vaccine efficacy due to the inherent resistance of approximately 60% of unvaccinated cats to development of persistent antigenemia after FeLV challenge (47).

$$PF = \frac{\text{Incidence of Persistent Antigenemia in Controls} - \text{Incidence of Persistent Antigenemia in Vaccinates}}{\text{Incidence of Persistent Antigenemia in Controls}}$$

The PF for Vaccine A was 85.7%. The PF for Vaccine B was –23.8%.

Host:virus relationships defined using circulating p27 and viral DNA levels

In the original FeLV:host relationship classification scheme, FeLV-exposed animals that did not develop persistent antigenemia were identified as having experienced regressive infections. The results of the present study suggest that FeLV-exposed antigen-negative cats represent a spectrum of host:virus relationships.

The 5 FeLV-A/61E-inoculated cats in which neither p27 nor viral DNA were detected at any time were classified as having experienced *abortive infection* (Table 1.4 and Fig. 1.4). Four of these cats were vaccinated with Vaccine A and 1 with Vaccine B.

The 6 cats that never developed detectable antigenemia but in which transient or low persistent circulating viral DNA levels were detectable (median: 225 copies/10^6 PBMC; range: 0 to 30,854 copies/10^6 PBMC) were classified as having experienced *regressive infection.* Five of these cats were vaccinated with Vaccine A and 1 with Vaccine B. An initial low proviral burden detected at 4 weeks PC was no longer demonstrable by 8 weeks PC in two cats vaccinated with Vaccine A.

Transient antigenemia was demonstrated in 3 unvaccinated control cats that retained persistent moderate proviral loads in blood (median: 40,969 copies/10^6 PBMC;

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range: 860 to 328,249 copies/10^6 PBMC). These animals were classified as retaining *latent infection.*

Finally, twenty-one cats developed persistent antigenemia with concurrent persistent high circulating proviral burdens (median: 269,328 copies/10^6 PBMC; range: 7,330 to 2,224,869 copies/10^6 PBMC). These animals, as in previous classification schemes, were designated as having a *progressive infection.*

Using repeated measures-ANOVA and the Tukey-Kramer post-hoc test, statistically significant differences ($p<0.01$) in p27 values were identified between progressive vs. abortive, progressive vs. regressive, and progressive vs. latent infection. Statistically significant differences ($p<0.01$) in viral DNA burdens were present among all FeLV categories (with the exception of latent vs. progressive infection): abortive vs. regressive, abortive vs. latent, abortive vs. progressive, regressive vs. latent, and regressive vs. progressive infection.

Table 1.4. Putative categories for FeLV:host relationships in vaccinated and unvaccinated cats challenged with FeLV-A/61E.

Group	Response category				Total
	Abortive	Regressive	Latent	Progressive	
	Provirus (-) Antigen (-)	Provirus (+)* Antigen (-)	Provirus (++) Antigen (+)→(-)	Provirus (+++) Antigen (+)	
Vaccine A	4	5	0	1	10
Vaccine B	1	1	0	13	15
Control	0	0	3	7	10
Total	5	6	3	21	

* After detecting an initial low proviral load, two of the six cats with regressive infection did not have detectable provirus at 8 weeks post-challenge. Both cats received Vaccine A.

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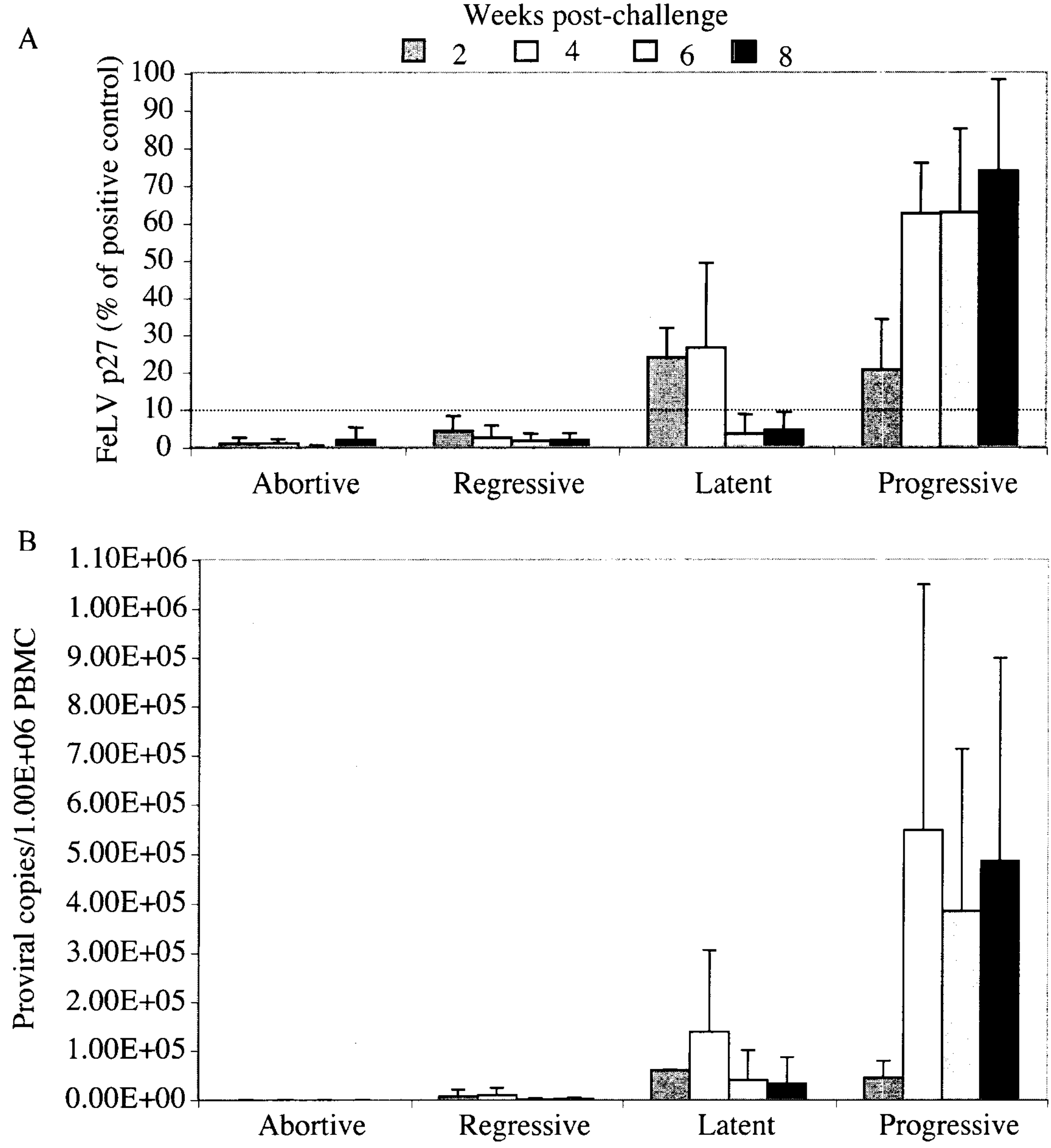

Fig. 1.4. Host:virus relationships defined using circulating p27 and viral DNA levels. FeLV-A/61E-infected cats classified as having experienced *abortive* infection never had detectable p27 (A) or viral DNA (B) in blood. In cats with *regressive* infection, circulating p27 was not detected but transient or persistent low viral DNA levels were detectable in blood. Cats considered to have *latent* infection developed transient antigenemia and retained moderate viral DNA levels in blood. Cats with *progressive* infection were persistently antigenemic and had persistent high circulating proviral burdens. Statistically significant differences ($p<0.01$) in p27 values were identified between progressive vs. abortive, progressive vs. regressive, and progressive vs. latent infection. Statistically significant differences ($p<0.01$) in proviral burdens were identified between abortive vs. regressive, abortive vs. latent, abortive vs. progressive, regressive vs. latent, and regressive vs. progressive infection. Mean ± SD are plotted. (A) The dotted line represents the threshold for positive results (≥10% of the positive control).

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Agreement and correlation between p27 and viral DNA detection

The kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance, between the p27 capture ELISA and the real-time PCR assay (Table 1.2). All samples that tested positive for p27 capsid antigen were positive by real-time PCR (76 samples from 23 cats). All samples with undetectable viral DNA (real-time PCR negative) had undetectable antigen (ELISA negative) (23 samples from 8 cats). No sample was positive by ELISA and negative by real-time PCR. However, 24 samples from 13 cats were positive by real-time PCR and negative by p27 capture. Thus, real-time PCR had greater sensitivity than p27 capture ELISA. The kappa statistic was 0.53 indicating a fair agreement between the two tests.

Pearson correlation coefficients were determined to assess the linear relationship between circulating p27 levels and PBMC viral DNA levels. After a Fisher's r to z transformation, p values were obtained. The correlation between ELISA and real-time PCR became progressively more concordant as infections became fully established as indicated by the following trend in time periods: 2 weeks PC $r = 0.761$, $p<0.01$; 4 weeks PC $r = 0.461$, $p<0.05$; 6 weeks PC $r = 0.555$, $p<0.01$; and 8 weeks PC $r = 0.640$, $p<0.01$. After splitting the data by category of FeLV infection, a more linear relationship between the assays appeared: abortive infection r = not applicable (no variability in the data); regressive infection $r = 0.831$, $p<0.01$; latent infection $r = 0.896$, $p<0.01$; and progressive infection $r = 0.409$, $p<0.01$.

Long-term outcome and host:virus relationships in 13 of the FeLV-challenged cats

Thirteen of the 35 cats studied above were available for necropsy after survival periods of 2 to 3.5 years. This cohort was comprised of: 5 cats from the Vaccine A

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group, 4 cats from the Vaccine B group, and 4 from the unvaccinated Control group (Table 1.1). Sera were analyzed for p27 capsid antigen via capture ELISA. PBMC, bone marrow (BM), spleen (SP), and mesenteric lymph node (MLN) from all 13 animals were analyzed for viral DNA via quantitative real-time PCR. In addition, thymus, tonsil, and retropharyngeal lymph node were available for the 5 cats vaccinated with Vaccine A.

Abortive infection

Three cats that received Vaccine A and were categorized as abortive infection (antigen negative/provirus negative) remained antigen and provirus negative in blood after a 2-year observation period (Fig. 1.5). Perhaps surprisingly, viral DNA was not detectable in the BM, SP, or MLN of these same animals. In addition, no viral DNA could be detected in thymus, tonsil, or retropharyngeal lymph node (data not shown). It would not be possible, therefore, to distinguish these animals from those never exposed to FeLV on the basis of antigen capture ELISA and viral DNA real-time PCR assay results alone.

Regressive infection

Two cats that received Vaccine A and were classified as regressive infection (antigen negative/low transient provirus) (Table 1.4) also remained antigen and provirus negative in blood nearly 2 years later. Similar to cats with abortive infections, viral DNA was not detected in BM, SP, or MLN, nor was it detected in thymus, tonsil, and retropharyngeal lymph node (data not shown). The 1 cat that received Vaccine B and was classified as regressive infection (antigen negative/persistent low proviral load)

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remained antigen negative. The relatively low PBMC viral DNA levels detected at 8 weeks PC (6866 ± 668 copies/10^6 PBMC) were retained 3 years later (44 ± 76 copies/10^6 PBMC) and these levels were similar to those detected in BM, SP and MLN.

Latent infection

The 1 unvaccinated control cat classified as latent infection (transient antigenemia/persistent moderate proviral load) had become p27-positive 3 years later. Viral DNA levels detected in PBMC of this animal were similar to BM, SP, and MLN although PBMC levels (919 ± 330 copies/10^6 PBMC) after 3 years were appreciably lower than those detected at 8 weeks PC (94,184 ± 4962 copies/10^6 PBMC).

Progressive infection

One cat that received Vaccine B and was considered to have progressive infection also remained unchanged 3 years later. The PBMC proviral load in this animal peaked at 4 weeks PC (518,096 ± 17,778 copies/10^6 PBMC), decreased by 8 weeks PC (7,330 ± 133 copies/10^6 PBMC), and remained relatively similar to the 8 week level 3 years later (196 ± 63 copies/10^6 PBMC). Proviral burdens in BM, SP, and MLN were similar to blood levels. Two cats that received Vaccine B and 3 unvaccinated control cats that were classified as progressive infections (antigen positive/persistent high proviral load) remained antigen-positive. The relatively high PBMC viral DNA levels detected at 8 weeks PC (639,174 ± 593,815 copies/10^6 PBMC) were retained 3 to 3.5 years later (2,143,280 ± 1,387,100 copies/10^6 PBMC) and these levels were similar to those detected in BM, SP and MLN.

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Viral DNA levels in circulating cells correlated with levels in tissues. Pearson correlation coefficients between circulating and tissue viral DNA levels and the p values after a Fisher's *r* to *z* transformation were PBMC vs. BM: $r = 0.559$, $p>0.05$; PBMC vs. SP: $r = 0.975$, $p<0.01$; and PBMC vs. MLN: $r = 0.823$, $p<0.01$.

In summary, it appeared in most instances the host:virus relationship became established by 8 weeks and was maintained for 2 to 3.5 years in blood and lymphoid tissues.

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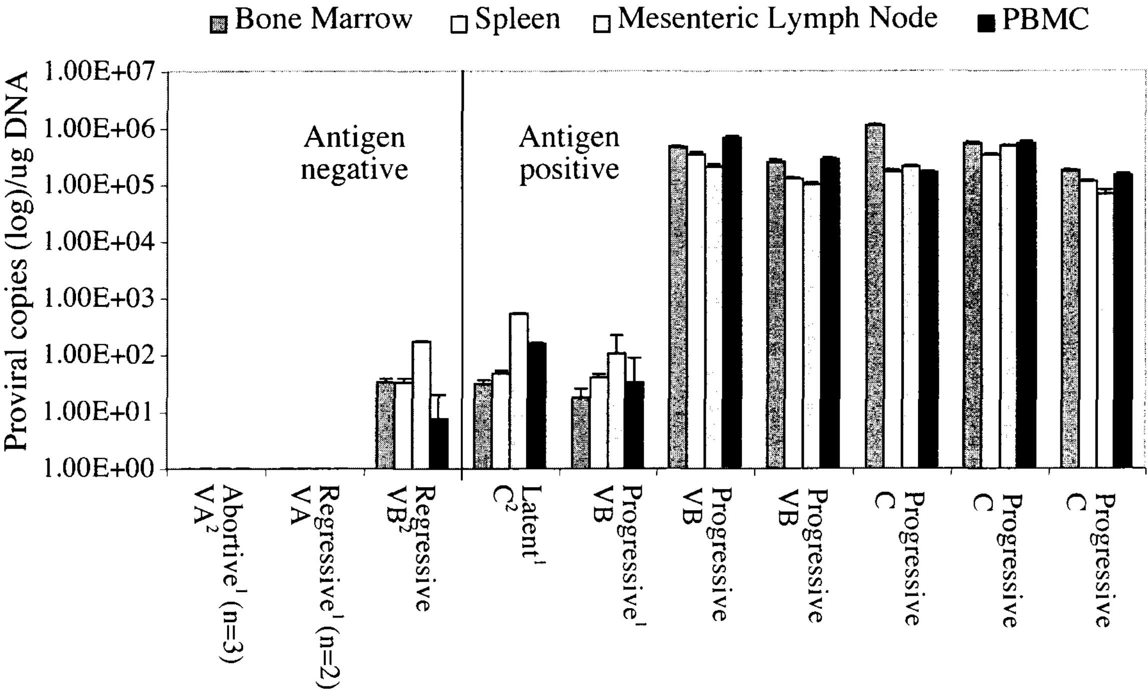

[1] Category of FeLV infection as classified by the p27 and viral DNA assays during the first 8 weeks post-challenge.

[2] Experimental group.

VA=Vaccine A, VB=Vaccine B, C=unvaccinated Control

Fig. 1.5. Early FeLV:host relationships were maintained for 2 - 3.5 years and proviral burdens in blood and tissues correlated. Thirteen of 35 cats were available for necropsy after long-term survival periods. Sera were analyzed for antigenemia via p27 capture ELISA. PBMC, BM, SP, and MLN were analyzed for viral DNA burden via quantitative real-time PCR. Three Vaccine A cats with abortive infection and 2 Vaccine A cats with regressive infection (transiently detectable viral DNA) remained p27 negative with undetectable viral DNA in PBMC, BM, SP, and MLN. Additional available tissues (thymus, tonsil, and retropharyngeal lymph node) also were negative for viral DNA (data not shown). One Vaccine B cat with regressive infection remained p27 negative despite retaining low viral DNA levels in PBMC, BM, SP, and MLN. The 1 unvaccinated control cat classified as latent infection became p27-positive with detectable viral DNA in PBMC, BM, SP, and MLN. The 3 Vaccine B cats and 3 unvaccinated control cats with progressive infection remained p27 positive with readily detectable viral DNA in PBMC, BM, SP, and MLN. Pearson correlation coefficients and p values between PBMC and tissues were PBMC vs. BM: $r = 0.559$, $p>0.05$; PBMC vs. SP: $r = 0.975$, $p<0.01$; and PBMC vs. MLN: $r = 0.823$, $p<0.01$. Mean ± SD are plotted.

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DISCUSSION

The primary aim of this study was to develop and validate a quantitative real-time DNA PCR assay to examine FeLV-vaccinated and unvaccinated cats for viral DNA sequences in circulating cells during the early phase of FeLV infection and both circulating cells and tissue during the late phase of FeLV infection. This assay was based on an FeLV U3 LTR sequence and proved to be reproducible, quantitative, sensitive, and specific for exogenous FeLV. The greater sensitivity of real-time PCR allowed detection of viral DNA in cats with undetectable antigenemia. This finding is consistent with recent studies of Hofmann-Lehmann *et al.* and Flynn *et al.* (10, 19). The current real-time PCR assay, while similar to that developed by Hofmann-Lehmann *et al.* (19), is based on FeLV-A/61E, the highly replication competent, non-acutely pathogenic component of the FeLV-FAIDS complex (9, 25, 39, 42). The U3 LTR region is conserved among FeLV subgroup A viruses, thus it is probable that detection of cross-isolates will occur using the present primer/probe set, although this issue was not addressed in the present study. While unintegrated viral DNA (UVD) is a characteristic of the FeLV-FAIDS strain, this method cannot distinguish between integrated provirus and UVD.

This would appear to be the first study assessing the efficacy of an FeLV vaccine using real-time PCR. Nine of the 10 cats which received Vaccine A (Fort Dodge Fel-O-Vax Lv-K®) were protected as indicated by the absence of circulating FeLV p27. Moreover, in 4 of the 9 protected vaccinates viral DNA was never detected in PBMC. The remaining 5 protected cats had either transient low (2 cats) or persistent low (3 cats)

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circulating viral DNA levels within the first 8 weeks PC. Importantly, viral DNA was not detectable in PBMC or lymphoid tissues from the 5 available animals, nearly 2 years after viral challenge. Previous studies examining the efficacy of Fel-O-Vax Lv-K® reported preventable fractions of 86% and 100% (23, 24, 32). Virus was not isolated from bone marrow cultures at 7 or 31 weeks post-challenge/exposure in these experiments (23, 24, 32). Results of the present study bolster these previous findings, as do those of Haffer *et al.*, (14) lending support to the tenet that successful immunity to retroviral infection can be obtained with immunoprophylaxis.

The greater sensitivity of real-time PCR allowed us to suggest more detailed FeLV:host relationship categories, which we designated as: abortive, regressive, latent, and progressive. Although it is certainly plausible that these categories of FeLV infection may be dynamic, especially the intermediate categories, we found these host:virus relationships became established by 8 weeks PC and were maintained for years in the limited sample of FeLV-challenged cats in the present study.

In the original FeLV:host relationship classification scheme, animals with abortive, regressive, and latent infection all would have been identified as regressive infection due to the lack of persistent antigenemia. In 1980-1982, it was subsequently discovered that at least some antigen-negative cats which experienced regressive infection retain latent FeLV infection in bone marrow (37, 48, 51). With the advent of PCR, this hypothesis was tested using antigen-negative cats with suspected latent infections, however, no viral DNA sequences were amplified from blood or bone marrow cells (18, 28, 38). Thus, it was proposed that these antigen-negative cats did not harbor latent virus in the sites examined. The results of the present study suggest that neither

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scenario is absolute. Rather, FeLV-exposed antigen-negative cats represent a spectrum of host:virus relationships wherein some animals appear to eliminate infected cells in circulation and tissues while some maintain a low to moderate level of infected cells. Reactivation is possible in the latter animals.

We hypothesize that cats with *abortive* infection produced effective early host immune responses which abrogate viral replication and eliminate FeLV-infected cells. This is inconsistent with the hypothesis that *all* FeLV-exposed antigen-negative cats harbor a reservoir of infected cells in some hemolymphatic tissue. It remains possible, though not probable in our view, that such animals harbor sequestered FeLV in tissues not examined. It is also possible that the real-time PCR assay is not sufficiently sensitive to detect extraordinarily low proviral levels, as has been proposed to occur in people who are repeatedly exposed to human immunodeficiency virus yet remain seronegative (56). Our present observations bolster the contention that some individuals can resist retroviral infection without conventional evidence of infection.

We propose that cats with *regressive* infection successfully contain viral replication despite retaining a low level of FeLV-infected cells in circulation and tissues. Some of these animals even eliminate these infected cells and go on to resemble cats with abortive infections. This supports the hypothesis that *some* FeLV-exposed antigen-negative cats can maintain populations of non-productive, infected cells. Our results also demonstrate that these cats harbor viral DNA in circulation and lymphoid tissues in addition to bone marrow. While reactivation of regressive infection may be possible, this was not detected in the present study. Overall, the present study suggests a more likely outcome of eventual elimination or extinction of infected cells.

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We propose in cats classified as *latent* infection that delayed containment of viral replication occurs resulting in a moderate proviral residuum. As a corollary, if host immune containment wanes, viral reactivation becomes more likely. This is consistent with the tenet that *some* FeLV-exposed antigen-negative cats can maintain cell populations harboring replication-competent latent FeLV capable of reactivation.

We assume that residual viral DNA detected by real-time PCR could represent intact provirus or replication-defective sequences. Previous studies have reported that non-viremic cats from which FeLV was isolated from cultured BM cells did not horizontally transmit FeLV (37, 43, 45). However, vertical transmission to offspring from similar animals also has been reported (44, 45). Additional studies are needed to assess the state and fate of viral DNA in latently infected cats. Such issues are pertinent to use of FeLV antigen-negative cats for blood donation, tissue transplants, and adoptions, as well as to the use of therapeutic immunosuppressive drugs in antigen-negative cats (8, 12, 40, 41).

That effective containment of human immunodeficiency virus may be possible is inferred by long-term nonprogression in HIV-infected individuals and apparent resistance to infection in highly HIV-exposed seronegative individuals. Genetic, virological, and immunological factors all likely play a role in HIV containment (7, 17, 33, 52). Animal models present unique opportunities to prospectively examine the initial events in immunopathogenesis. Further examination of the early immune responses which determine effective vs. ineffective containment of FeLV infection and better characterization of the latent viral state would provide valuable insights into retroviral pathogenesis and resistance overall.

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CHAPTER 2

DEVELOPMENT AND APPLICATION OF A QUANTITATIVE REAL-TIME PCR ASSAY TO DETECT FELINE LEUKEMIA VIRUS RNA

ABSTRACT

We previously defined four categories of FeLV infection, designated as abortive, regressive, latent, and progressive. To determine if detectable viral DNA is transcriptionally active in the absence of antigenemia, we developed and validated a real-time viral RNA qPCR assay. This assay proved to be highly sensitive, specific, reproducible, and allowed reliable quantitation. We then applied this methodology, together with real-time DNA qPCR and p27 capsid antigen capture ELISA, to examine cats challenged with FeLV. We found that circulating viral RNA and DNA levels were highly correlated and the assays were almost in perfect agreement. This indicates that the vast majority of viral DNA is transcriptionally active, even in the absence of antigenemia. The real-time qPCR assays are more sensitive than the most commonly used FeLV diagnostic assay, the p27 capsid antigen capture ELISA. Application of qPCR assays may add greater depth in understanding of FeLV-host relationships.

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INTRODUCTION

Feline leukemia virus (FeLV) was first identified as a naturally occurring viral infection of cats more than 40 years ago by electron microscopy (EM) (23, 24). Since that time, advances in sensitivity, specificity, and speed of FeLV diagnosis have prompted greater insight into the complexity in the FeLV-host relationship. The clone 81 cell line used to detect infectious virus (VI) by focus formation (5, 7) and the direct immunofluorescent antibody (IFA) assay used to detect intracellular *gag* proteins (12, 13) provided the first major insights into FeLV detection and led to recognition of progressive and regressive host-virus relationships (20). Development of an antigen capture ELISA using monoclonal antibodies directed against different epitopes of the p27 capsid protein (26) provided a rapid and sensitive diagnostic assay applicable to testing animals on site at low cost.

Use of the VI, IFA, and ELISA assays in combination led to recognition of cats with 'discordant' results (14, 22, 27, 28), which in turn provided the first indication of more complex host-virus relationships among FeLV infections (18). With the advent of molecular diagnostics, conventional PCR to detect FeLV DNA was employed in attempt to better understand these discrepant results (15, 21, 30), but the assay did not appear to have increased sensitivity or specificity.

We, and other investigators, developed a quantitative real-time FeLV DNA PCR assay (qPCR) as means to both diagnose FeLV infection and seek further insight into the determinants of the virus-host relationship (8, 16, 34, 35). When we applied DNA qPCR, together with the antigen capture ELISA, in vaccinated and unvaccinated cats challenged

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with FeLV-A/61E, we identified a spectrum of host-virus relationships in FeLV-exposed cats which did not develop persistent antigenemia (35). Some animals experienced *abortive* infections—i.e. infections marked not only by undetectable antigenemia at all timepoints, but also the absence of infected cells in both circulation and tissues. Because these animals appeared not to maintain a tissue reservoir, it would be impossible to distinguish these animals from those never exposed to FeLV. By contrast, two additional groups of FeLV-exposed cats which did not develop persistent antigenemia were found to maintain either low or moderate levels of infected cells in circulation and tissues—and were designated as having experienced *regressive* or *latent* infections, respectively, depending on whether transient antigenemia was recognized. Finally, cats which developed overt persistent antigenemia with persistent high circulating and tissue viral DNA burdens represented those with *progressive* infection.

The finding of previously covert viral DNA in some cats which ostensibly totally resisted FeLV infection was not restricted to FeLV-A/61E, as other investigators found a similar phenomenon in cats challenged with the FeLV-A/Glasgow-1 (4, 8-10, 16, 17, 34). It remained unknown, however, whether the viral DNA we detected by our qPCR assay represented intact, replication-competent provirus or replication-defective viral DNA sequences. To determine whether this viral DNA is transcriptionally active in the absence of antigenemia we developed a qPCR assay to quantitate FeLV RNA in feline plasma. The validation and application of this assay is described here.

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MATERIALS AND METHODS

Experimental animals and challenge virus

Forty specific-pathogen-free (SPF) cats were obtained from Harlan Sprague Dawley, Inc. (Mt. Horeb, WI). The cats were randomly housed up to 5 cats per enclosure at Charmany Instructional Facility at the University of Wisconsin-Madison School of Veterinary Medicine (Madison, WI). The animals were housed in accordance with the university animal care and use committee regulations. At 34-35 weeks of age, all cats were challenged intraperitoneally with 200 μL of 5 X 10^4 $TCID_{50}$/mL FeLV-A/61E. Cats were observed twice daily for signs of illness after virus inoculation. Sample collections were performed on cats anesthetized with an intramuscular administration of ketamine hydrochloride (11 mg/kg).

Sample collection and processing

Blood samples were collected at challenge and every week thereafter through 8 weeks post-challenge (PC). Whole blood was shipped overnight on ice to Colorado State University (Ft. Collins, CO) where they were immediately processed upon arrival. Buffy coat cell pellets were stored at -70°C until analysis for FeLV DNA by qPCR. Plasma samples were separated into 1 mL aliquots and stored at -70°C until analysis for FeLV RNA by qPCR and FeLV p27 capsid antigen by capture ELISA.

DNA was extracted from buffy coat cells using a QIAamp DNA blood mini kit (QIAGEN, Inc., Valencia, CA), eluted in 100 μL of elution buffer, and DNA concentrations determined spectrophotometrically. RNA was extracted from 140 μL of

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plasma using a QIAamp viral RNA mini kit (QIAGEN). On-column digestion of DNA during RNA purification was performed using the RNase-free DNase set (QIAGEN). The RNA was eluted in 80 μL of elution buffer.

Primers and probe for RNA and DNA qPCR assays

We designed a primer/probe set to amplify exogenous and not endogenous FeLV sequences within the U3 region of FeLV-A/61E (1, 3) as previously described (35). These primers and probe were used to detect both FeLV RNA and FeLV DNA.

RNA standard preparation for absolute quantification

The plasmid p61E-FeLV, an *Eco*RI fragment containing the full-length FeLV-A/61E provirus subcloned into pUC18 (6, 32), was used to construct an RNA standard. This plasmid was provided as ampicillin-resistant transformed *E. coli* JM109 cells through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. James Mullins. The transformed *E. coli* cells were grown on LB media containing 50 μg/mL ampicillin. Plasmid DNA was isolated from the bacterial cells using the QIAfilter™ plasmid midi kit (QIAGEN). The plasmid was then double digested with *Eco*RI and *Bgl*II. The 1909 bp fragment was confirmed by agarose gel electrophoresis with GelStar® (Cambrex, Corp., East Rutherford, NJ) staining and gel purified using the QIAquick® gel extraction kit (QIAGEN). The pGEM®-3Z vector (Promega, Corp., Madison, WI) was double digested with *Eco*RI and *Bam*HI and the linearized vector, minus 21 bp, was confirmed by agarose gel electrophoresis. The purified 1909 bp fragment from p61E-FeLV was directly ligated into the linearized

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pGEM®-3Z using a Quick Ligation™ kit (New England BioLabs, Inc., Ipswich, MA). The constructs were transformed into chemically competent *E. coli* JM109 cells (Promega) and the cells were grown on LB media with 50 μg/mL ampicillin using blue/white screening. Plasmid DNA was isolated from the bacterial cells, double digested with *Eco*RI and *Hind*III, and the insert confirmed by agarose gel electrophoresis. The recombinant plasmid (named pGEM-3Z-61E) was sequenced by Davis Sequencing, Inc. (Davis, CA) to verify the insert orientation and length, and the primer/probe target site within the U3 region.

The pGEM-3Z-61E plasmid was linearized with *Hind*III and purified by the QIAquick® gel extraction kit. RNA transcripts (1943 nt) were produced via *in vitro* transcription using the T7 RiboMAX™ express large scale RNA production system (Promega). Residual plasmid DNA was removed using one RQ1 RNase-free DNase (Promega) and two TURBO DNase™ (Ambion, Inc., Austin, TX) treatments. After each DNase treatment, the resulting RNA transcripts were purified using the MEGAclear™ kit (Ambion). The absence of contaminating DNA template was confirmed by RNA qPCR of the RNA standard with and without the addition of reverse transcriptase (RT) to the reaction. The RNA standard copy number was calculated from optical density measurements at 260 nm. The RNA standard was diluted to 10^9 copies/μL in THE RNA storage solution (Ambion) with 30 ng/μL transfer RNA (Sigma-Aldrich, Corp., St. Louis, MO) as a carrier. This RNA stock was aliquoted and frozen immediately at –70°C. Each aliquot was used for making a single-use 10-fold dilution series. The starting quantities of the samples were determined by comparing the threshold cycle (C_T) value of the samples' RNA with the standard curve of the co-amplified standard template RNA.

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Detection of FeLV RNA in plasma by a one-tube qPCR assay

The 25 μL one-tube reaction consisted of 400 nM of each primer, 80 nM of fluorogenic probe, 12.5 μL of TaqMan® One-Step RT-PCR Master Mix (Applied Biosystems), 0.625 μL of MultiScribe™ Reverse Transcriptase and RNase Inhibitor Mix (Applied Biosystems), 2.875 μL of PCR-grade H_2O, and 5 μL of sample or RNA standard. The master mix was supplied at a 2X concentration and contained AmpliTaq® Gold DNA Polymerase, dNTPs with dUTP, and optimized buffer components. Reactions were performed in triplicate using an iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Every reaction plate contained a negative control (FeLV-naïve SPF cat RNA), a template control (no RNA, PCR-grade H_2O), and an extraction control (extracted PCR-grade H_2O). Thermal cycling conditions were 30 minutes at 48°C for the RT reaction, 10 minutes at 95°C to activate AmpliTaq® Gold DNA Polymerase and to denature the template cDNA, followed by 40 cycles of 15 seconds at 95°C for denaturation and 60 seconds at 60°C for annealing/extension.

Analytical specificity and sensitivity of RNA qPCR assay

Following agarose gel electrophoresis confirmation, the 68 bp PCR products from two separate reactions were sequenced to verify analytical specificity. The TOPO TA Cloning® kit (with pCR®2.1-TOPO® vector) (Invitrogen, Corp., Carlsbad, CA) was used for cloning the amplicons prior to sequencing. Briefly, the PCR products were directly ligated into the linearized pCR®2.1-TOPO® vector, the constructs were transformed into One Shot® TOP 10 chemically competent *E. coli* cells (Invitrogen), and the cells grown on LB media with 50 μg/mL ampicillin using blue/white screening.

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Plasmid DNA was isolated from the bacterial cells, linearized with *Eco*RI, and the insert confirmed by agarose gel electrophoresis. The cloned inserts were sequenced by Davis Sequencing. The sequences of the PCR products were then aligned with FeLV-A/61E using MacVector™ software version 7.0 for Macintosh, copyright 2000 (Oxford Molecular, Ltd., Madison, WI).

End-point dilution experiments of the RNA standard were performed to assess analytical sensitivity. A dilution series of 500, 100, 50, 10, 5, and 1 copies of the RNA standard, each in triplicate, was tested.

Amplification efficiency and reproducibility of RNA qPCR assay

To assess amplification efficiencies, serial dilutions (1:10, 1:100, and 1:1000) of plasma RNA from an experimentally FeLV-A/61E-infected cat and of the RNA standard were amplified in triplicate and the difference in the slopes (Δs) of the regression lines (C_T vs. dilution) was evaluated.

To assess assay reproducibility, dilutions of the RNA standard (5×10^7, 5×10^6, and 5×10^5 copies) and of RNA from an experimentally FeLV-A/61E-infected cat (neat, 1:10, and 1:100) were evaluated for within-run and between-run precision. Each dilution was run 10 times within the same reaction plate and between 10 different reaction plates to test the within-run and between-run precision, respectively. The coefficients of variations (CV) of the threshold cycles (C_T) were calculated: $CV(C_T)$.

Detection of FeLV DNA in buffy coat cells by qPCR assay

FeLV DNA was detected in buffy coat cells as previously described (35).

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Detection of FeLV p27 capsid antigen in plasma by capture ELISA

FeLV p27 capsid antigen was detected in plasma by capture ELISA using the monoclonal antibodies (mAbs) anti-p27 A2 and G3 (26) (kindly provided by Niels C. Pedersen; University of California, Davis, CA) as previously described with minor modifications (35). Briefly, we adjusted the concentration of the primary mAb, G3, to 0.25μg/well and the secondary horseradish peroxidase-conjugated mAb, A2, to 1:000. In addition, serum was assessed by the IDEXX SNAP® FeLV antigen diagnostic test (IDEXX Laboratories, Inc., Westbrook, ME). In rare instances of incongruous results, the most sensitive finding of the two assays was chosen.

Data analysis

The kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance, between the RNA and DNA qPCR assays and between the RNA qPCR assay and the p27 capture ELISA. Pearson correlation coefficients were determined to assess the linear relationship between viral RNA vs. DNA levels and between viral RNA vs. p27 levels. After a Fisher's r to z transformation, p values were obtained. A statistically significant difference between tests was considered to have occurred when a p value was < 0.05. Undetectable results by both qPCR assays were corrected to a value of one and then all viral RNA and DNA levels were log transformed. The Pearson correlation coefficient was performed using StatView® version 5.0.1 for Macintosh, copyright 1999 (Abacus Concepts, Inc., Berkeley, CA).

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RESULTS

RNA qPCR specificity

The analytical specificity of the FeLV RNA qPCR assay was confirmed by sequencing two amplicons after agarose gel confirmation. Using MacVector™ software, the amplicon sequence from the RNA standard was identical to that of FeLV-A/61E and the amplicon sequence from an FeLV-A/61E-infected cat contained one base mismatch. Plasma RNA from FeLV-naïve, SPF cats were consistently negative for FeLV RNA (43/43 samples from 9 cats). Consequently, diagnostic specificity was 100% in FeLV-A/61E-infected animals.

RNA qPCR sensitivity

The analytical sensitivity of the FeLV RNA qPCR assay was assessed in end-point dilution experiments. These studies consistently detected 10 copies and inconsistently detected 5 copies of the RNA standard (data not shown). Negative samples never crossed threshold: negative control (FeLV-naïve SPF cat RNA), template control (no RNA, PCR-grade H_2O), extraction control (extracted PCR-grade H_2O), DNA control (no RT added to RNA standard), and samples containing 1 copy of the RNA standard. All FeLV-A/61E-infected cats that tested positive for p27 capsid antigen also were positive by qPCR (88/88 samples from 19 cats) (Table 2.1). Thus, diagnostic sensitivity in the animals studied was 100%.

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Table 2.1.
RNA qPCR vs. DNA qPCR and p27 capsid capture ELISA for FeLV detection

		RNA qPCR		
		(+)	(-)	
DNA qPCR	(+)	128	22	Kappa value = 0.82 (almost perfect agreement)
	(-)	2	112	
p27 ELISA	(+)	88	0	Kappa value = 0.68 (substantial agreement)
	(-)	42	134	

RNA qPCR linearity

The linear range of the RNA standard curve was evaluated. Amplification of ten-fold serial dilutions starting at 5 X 10^7 copies and ending at 5 X10^1 copies of the RNA standard from 16 independent experiments demonstrated linearity over 6 orders of magnitude, generated a standard curve correlation coefficient of 0.999, and produced an amplification efficiency (25) of 105.8% (Fig. 2.1).

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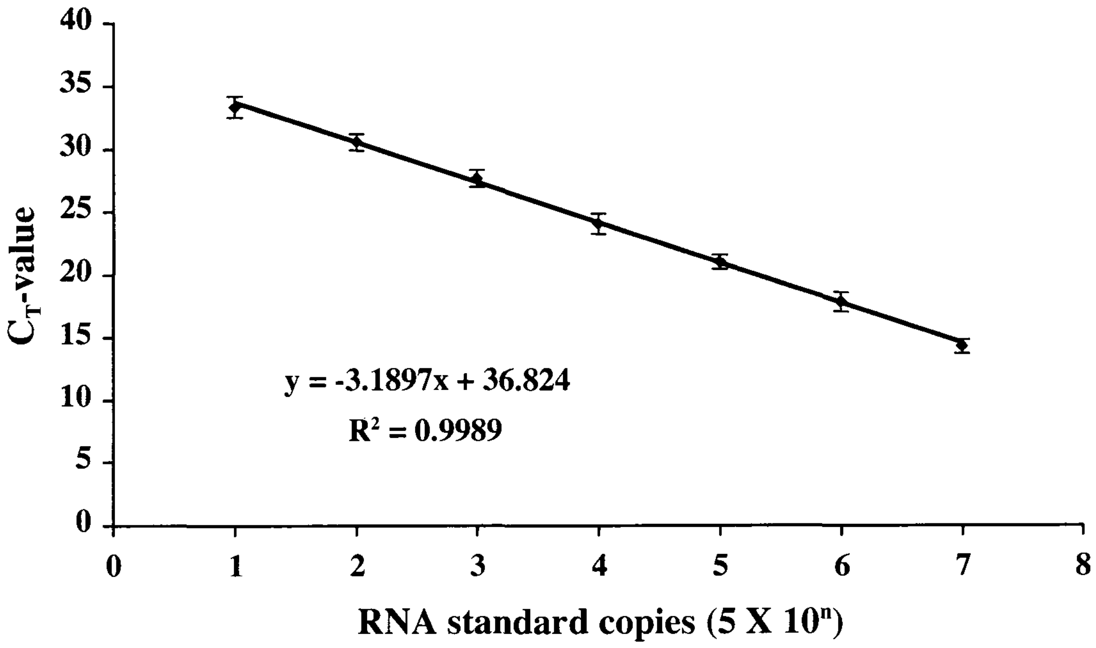

Fig. 2.1. Linearity and sensitivity of quantitative real-time FeLV RNA PCR. Standard curve of the RNA standard is linear. Amplification of ten-fold serial dilutions between 5 X 10^7 copies to 5 X10^1 copies of the standard demonstrated linearity over six orders of magnitude, generated a standard curve correlation coefficient of 0.9989, and produced an amplification efficiency of 105.8%. Ten copies of the standard are consistently detected. Mean ± SD for 16 independent experiments are plotted.

RNA qPCR amplification efficiency

The amplification efficiencies of FeLV-A/61E-infected cat RNA and the RNA standard were compared to validate quantification using the RNA standard. Equivalent amplification efficiencies are indicated by regression line slopes (s) with less than 0.1 difference (Δs) (11). The observed amplification efficiencies of the target RNA (s=3.17, R^2=0.993) vs. the RNA standard (s=3.24, R^2=0.997) had a Δs=0.07 (Fig. 2.2). Thus, quantification using the RNA standard was expected to be valid.

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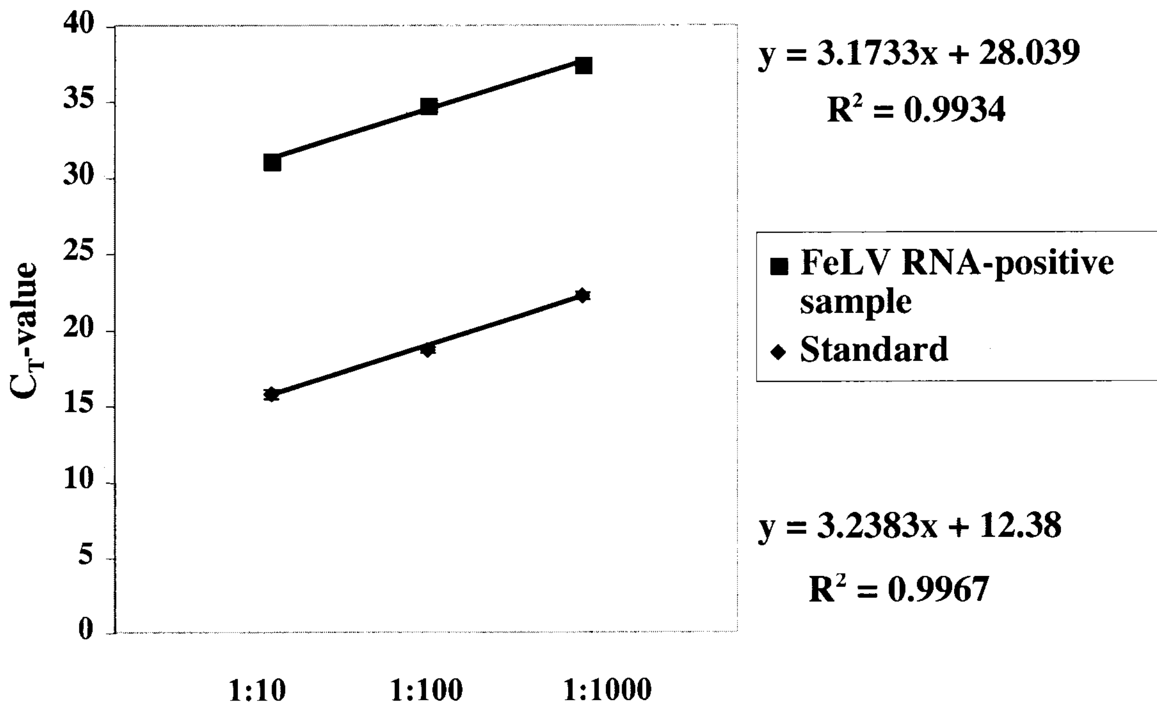

Fig. 2.2. Amplification efficiency comparison to validate real-time RNA PCR quantification. Serial dilutions (1:10, 1:100, and 1:1000) of plasma RNA from an FeLV-A/61E-infected cat and of the RNA standard were amplified. Amplification efficiencies of the FeLV-positive RNA and the RNA standard were approximately equal (Δs=0.07) demonstrating that quantification using the RNA standard is expected to be valid. Mean ± SD are plotted.

RNA qPCR reproducibility

The within-run and between-run precision of the FeLV RNA qPCR assay was evaluated. Several dilutions of the RNA standard and of FeLV-A/61E-infected cat RNA were amplified 10 times within the same reaction plate and between 10 different reaction plates. The threshold cycle coefficients of variation, CV(C_T), for the within-run precision was 0.93 to 1.81% and the CV(C_T) for the between-run precision was 0.9 to 2.18% (Table 2.2).

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Table 2.2.
RNA qPCR coefficients of variations (%) of within-run and between-run precision.

	RNA standard (copies)			FeLV-positive RNA (dilution)		
	5×10^7	5×10^6	5×10^5	neat	1:10	1:100
CV (C_T) within-run	1.81	1.21	1.44	1.08	1.03	.93
CV(C_T) between-run	1.94	2.13	2.06	2.18	1.19	.90

RNA qPCR agreement and correlation with DNA qPCR and antigen capture ELISA

The kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance, between the RNA qPCR assay and the DNA qPCR assay and between the RNA qPCR assay and the p27 capture ELISA (Table 2.1). The majority of samples tested by RNA and DNA qPCR had identical results; 128 samples from 23 cats were positive for viral RNA and DNA while 112 samples from 19 cats were negative for viral RNA and DNA. However, 22 samples from 9 cats had circulating viral DNA but undetectable viral RNA. Surprisingly, 2 samples from 2 cats were found to be negative by DNA qPCR but positive by RNA qPCR. The kappa statistic was 0.82 indicating an almost perfect agreement between the two tests. All samples that tested positive for p27 capsid antigen were positive for viral RNA (88 samples from 19 cats). All samples with undetectable viral RNA had undetectable antigen (134 samples from 25 cats). In no sample in which viral RNA was undetected was viral antigen detected. However, 42 samples from 22 cats were positive by RNA qPCR and negative by p27 capture. Thus, RNA qPCR had a greater sensitivity than p27 capture ELISA. The kappa statistic was 0.68, indicating a substantial agreement between the two tests.

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Pearson correlation coefficients were determined to assess the linear relationship between circulating viral RNA levels, DNA levels, and p27 levels. After a Fisher's r to z transformation, p values were obtained. An extremely strong linear relationship between viral RNA and DNA levels was identified; r = 0.939, p < 0.0001 (Fig. 2.3). Surprisingly, the p27 levels correlated more strongly with the viral DNA load (r = 0.776, p < 0.0001) than with the viral RNA load (r = 0.428, p < 0.0001) (data not shown).

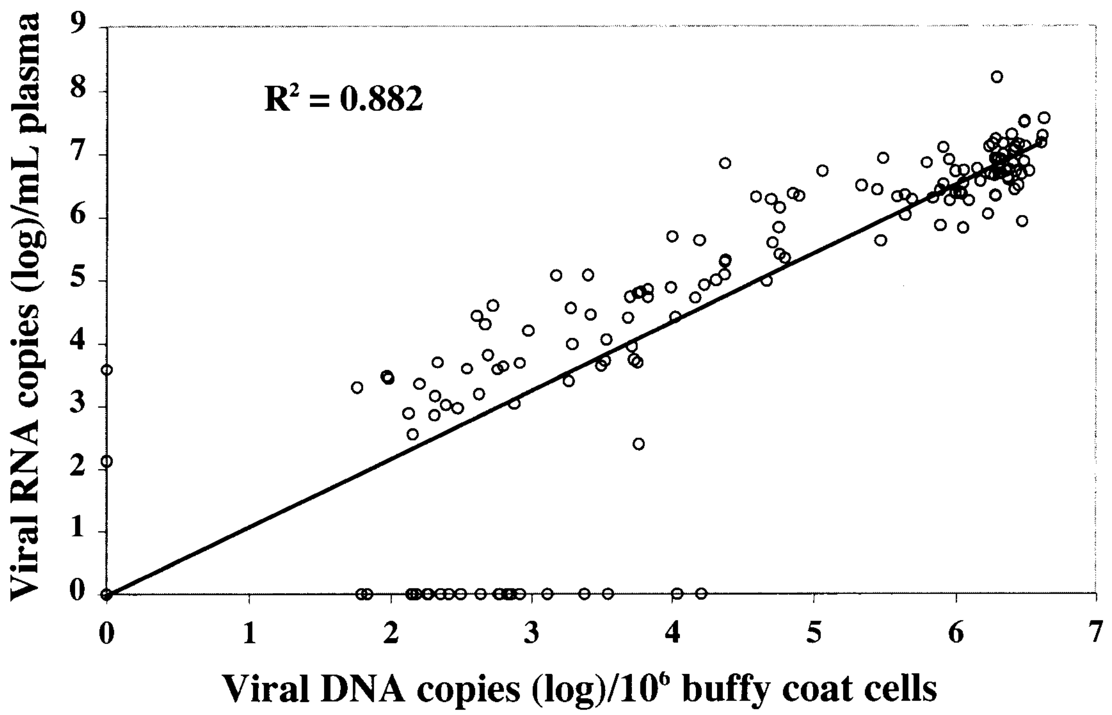

Fig. 2.3. Correlation of FeLV RNA vs. DNA levels. Plasma and buffy coat cells collected at challenge and weekly thereafter through 8 weeks PC from 40 cats were analyzed for viral RNA and DNA, respectively, by qPCR assays. The Pearson correlation coefficient was determined. Viral RNA levels strongly correlated with viral DNA levels (r = 0.939, p < 0.0001) thus, a positive linear relationship existed.

DISCUSSION

Development of the direct immunofluorescent antibody assay by Hardy *et al.* (12, 13) to detect FeLV *gag* antigens within leukocytes and platelets on blood films made

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possible elucidation of the contagious transmission of FeLV. The subsequent antigen capture ELISA of Lutz *et al.* (26) detected extracellular p27 capsid antigen in plasma or sera and made possible the rapid in-house detection of FeLV infection. Usually results of these assays correlated. However, in some animals extracellular (serum, plasma) viral antigen was detected in the absence of detectable antigen in circulating cells and/or infectious virus in plasma (14, 22, 27, 28). Potential reasons for the 'discordant' results could reflect assay sensitivities or specificities, sample handling, and assay expertise. In addition, the basis for discrepancies could lie in the biology of FeLV (33), including antigen detected in plasma before the stage of bone marrow cell infection, presence of latent virus capable of producing antigen but not infectious virus, or virus being produced only in atypical sequestered foci.

Application of qPCR to detect FeLV DNA in conjunction with p27 capsid capture ELISA has resulted in diagnostic sensitivity of 100% (4, 8-10, 16, 17, 34, 35). In addition we, and others, found animals in which viral DNA was detected in the absence of detectable antigenemia, demonstrating that qPCR sensitivity was greater than p27 ELISA and that viral DNA burden was an accurate predictor of FeLV infection outcome. However, these DNA qPCR assays did not differentiate unintegrated viral DNA from integrated provirus. Work by Cattori *et al.* (4) addressed this issue by designing methods to detect integration of FeLV DNA into the host genome. These investigators demonstrated that proviral integration occurs not only in cats with persistent antigenemia, but also in cats without detectable antigenemia and lower proviral burdens.

The primary purpose of the present work was to develop an assay to determine whether viral DNA detected in non-antigenemic cats represented integrated provirus that

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was transcriptionally active. We based the RNA qPCR assay on the U3 sequence of FeLV-A/61E (the replication competent, non-acutely pathogenic component of the FeLV-FAIDS complex) (6, 19, 31, 32) both to increase the probability of detecting other strains of FeLV-A, owing to the conservation of the U3 region, (1, 3) and to minimize the potential for detection of RNA that may under some circumstances be transcribed from the endogenous *env* sequences present in the feline genome (2, 29). Our assay proved to be highly sensitive, specific, reproducible, and allowed reliable quantitation. Additionally, qPCR enables a high throughput of samples in a very short amount of time. The detection limit of 5 – 10 viral copies per qPCR reaction corresponds to roughly 575 – 1150 viral copies per mL plasma, comparable with the sensitivity of the RNA qPCR assay described by Tandon and colleagues (34). While we detected viral RNA in all p27-positive cats in our study, Tandon *et al.* (34) found viral RNA to be undetectable in 3 of 41 antigenemic cats.

Agreement between the RNA and DNA qPCR assays was very high (Kappa = 0.82) with 240 samples from a total of 264 samples having concordant results (either both positive or both negative). In addition, a strong linear relationship between the two assays was identified ($r = 0.939$, $p < 0.0001$). A similar correlation was found by Tandon *et al.* (34). Consistent with other investigators, we identified 22 samples which had detectable, albeit low, viral DNA levels and undetectable viral RNA (9, 10, 17, 34). Several possible explanations for discordant RNA and DNA qPCR results exist. Most likely is simply that RNA qPCR is less sensitive than DNA qPCR. Viral RNA in plasma may be less stable than viral DNA within cells although the viral envelope should protect FeLV RNA from nucleases. Presuming detectable viral DNA is integrated, it is possible

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that the provirus is replication-defective or in a transcriptionally silent (latent) state. Because the diagnostic specificity was previously identified at 100%, it is unlikely that these DNA qPCR results are false positives (35). Surprisingly, we also identified 2 samples in which low viral RNA levels were detected without detectable viral DNA; one sample was at 1 week and one was at week 8 PC. This phenomenon was also observed by Hofmann-Lehmann (17). The 1-week PC sample may represent the detection of a primary cell-free viremia whereby the virus is replicating in local lymphoid tissue but lymphocytes containing viral DNA are not yet circulating. The terminal 8-week PC sample could represent cell-free viremia possibly due to atypical sequestered foci of FeLV infected cells. Because the diagnostic specificity of our RNA qPCR assay was 100%, it is unlikely this sample was falsely positive.

Both the DNA and RNA qPCR assays were more sensitive than the most commonly used clinical FeLV diagnostic assay, the capsid antigen capture ELISA. Although the agreement between RNA qPCR and antigen capture ELISA was substantial (Kappa = 0.68), in 42 samples viral RNA was detected in the absence of antigenemia. In contrast to the experience of Tandon *et al.* we found the p27 ELISA to be more strongly correlated with DNA qPCR ($r = 0.776$) than with RNA qPCR ($r = 0.428$) (34).

The RNA qPCR results are consistent with the concept that at least part of the detected FeLV DNA detected in previous work is integrated and initiates a transcriptionally active infection, even in the absence of detectable antigenemia. This infers that infectious virus and p27 antigen are produced but below assay sensitivity. However, the clinical relevance of detectable nucleic acids without detectable antigen or infectious virus remains unknown. While we have shown that FeLV-host relationships

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established by 8 weeks PC can remain unchanged for years, such "latently infected" cats may serve as a risk to susceptible cats. Application of qPCR assays may add greater depth in understanding of FeLV-host relationships.

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CHAPTER 3

VACCINATION AGAINST FELINE LEUKEMIA VIRUS CAN INDUCE STERILIZING IMMUNITY WITH MINIMAL VIRUS NEUTRALIZING ANTIBODY

ABSTRACT

A fraction of cats exposed to feline leukemia virus (FeLV) effectively contain virus and resist persistent antigenemia/viremia. Using real-time PCR (qPCR) to quantitate circulating viral DNA levels, we previously detected persistent FeLV DNA in the blood cells of non-antigenemic cats that had ostensibly resisted viral challenge. To determine whether this viral DNA is transcriptionally active in the absence of antigenemia, we developed and applied a reverse transcriptase qPCR assay to detect plasma-borne viral RNA. Here we have examined 40 FeLV-vaccinated and unvaccinated cats challenged with FeLV and demonstrated an almost perfect agreement and a strong linear relationship between viral DNA and RNA levels, inferring that detected FeLV DNA is both integrated and transcriptionally active. However, only high levels of viral DNA and RNA were associated with detectable infectious virus, connoting either limits in the viral infectivity assay and/or that a substantial fraction of viral particles are

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defective. These studies also demonstrated that two whole inactivated virus (WIV) adjuvanted FeLV vaccines (Fel-O-Vax Lv-K® by Fort Dodge Animal Health and FEVAXYN FeLV® by Schering-Plough Animal Health Corporation) provided very effective protection against FeLV challenge. In nearly every recipient of these vaccines, neither viral DNA, RNA, antigen, nor infectious virus could be detected in blood. Moreover, effective viral containment occurred despite a weak virus neutralizing antibody response. The above findings reinforce the precept of FeLV infection as a model of the early immune responses that determine effective vs. ineffective containment of retroviral infections, and hold valuable insights into immunoprevention and therapy.

INTRODUCTION

Feline leukemia virus (FeLV) was first identified as a naturally occurring viral infection of cats more than 40 years ago (37, 38, 61). The primary route of transmission of this gammaretrovirus is horizontally through saliva (12, 17, 18, 32). The pathogenic effects induced by FeLV are both cytoproliferative (e.g. lymphoma or myeloproliferative disorder) as well as cytosuppressive (e.g. immunodeficiency or myelosuppression) [reviewed by Hoover and Mullins (27)].

Diagnosis of FeLV infection has necessarily been based on assays detecting substantial FeLV viremia by cell culture infectivity assay (5, 8), intracellular antigen in leukocytes by immunofluorescence (IFA) (18, 19), or extracellular (serum, plasma) antigenemia by antigen capture ELISA (43). However, use of these assays in combination led to identification of animals with discrepant results; extracellular viral

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antigen was detected in the absence of detectable antigen in circulating cells and/or infectious virus in plasma (20, 35, 45, 46). The concomitant recognition of latent FeLV infections in antigen-negative cats gave the first intimation that more nuanced host:virus relationships were in play and/or the available assays were simply limited by sensitivity (47, 58, 63).

We have used a quantitative real-time PCR (qPCR) assay to examine host:virus relationships in vaccinated and unvaccinated cats challenged with FeLV-A/61E. We detected FeLV DNA, in both circulation and tissues, in the absence of detectable antigenemia (71). These findings, and those of Hoffmann-Lehmann and colleagues (10, 25, 68), demonstrated that qPCR sensitivity was greater than p27 ELISA. Moreover, we revealed 4 categories of FeLV:host relationships: 1) undetectable antigenemia and viral DNA, 2) undetectable antigenemia and low viral DNA levels, 3) transient antigenemia and moderate viral DNA levels, 4) persistent antigenemia and high viral DNA levels. qPCR detection of occult viral DNA in some cats which ostensibly totally resisted FeLV infection did not differentiate unintegrated viral DNA from integrated provirus. However, work by Cattori *et al.* (3) using methods to detect integration of FeLV DNA into the host genome demonstrated that proviral integration occurs not only in cats with persistent antigenemia, but also in cats without detectable antigenemia and lower proviral burdens.

We next wished to determine whether FeLV DNA detected by our qPCR assay represented intact, replication-competent provirus or viral DNA that was either not integrated into the host cell genome or was otherwise replication-defective. We hypothesized the FeLV infection was indeed productive and the absence of antigenemia

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was due to limits in sensitivity of the antigen detection by ELISA. The purpose of this study therefore, was to determine if the detectable viral DNA was transcriptionally active. To detect extracellular viral RNA we developed a reverse transcriptase qPCR assay to quantitate FeLV RNA in feline plasma and performed viral infectivity assays to determine whether detectable RNA represented infectious virus.

An abundance of vaccine trials have evaluated commercial vaccine efficacy, reviewed by Loar (42) and Sparkes (67). Because these studies have used varying challenge viruses, dosages, routes, and ages of vaccinates, it is difficult to make meaningful comparisons. Using our qPCR assay, we demonstrated that the whole inactivated virus (WIV) vaccine Fel-O-Vax Lv-K® (Fort Dodge Animal Health) provided protection against FeLV challenge (71). This was consistent with previous studies which collectively supported a similar conclusion (23, 28, 29, 40, 53). To our knowledge however, an investigation of multiple commercially available (in the USA) FeLV vaccines has not been performed using modern viral nucleic acid detection methods. Here we have examined viral DNA, viral RNA, p27 Gag antigen, and infectious virus in cats immunized with various FeLV vaccines and challenged with the prototype FeLV subgroup A strain FeLV-A/61E. We also report virus neutralizing antibody levels in these animals to determine whether a humoral immune response was associated with contained viral infection.

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MATERIALS AND METHODS

Cats

Forty specific-pathogen-free (SPF) cats were obtained from a commercial vendor (Harlan Sprague Dawley, Inc., Mt. Horeb, WI). The cats were randomly housed up to 5 cats per enclosure at Harlan Sprague Dawley during the immunization phase of the experiment. Prior to virus challenge, they were transferred to Charmany Instructional Facility at the University of Wisconsin-Madison School of Veterinary Medicine (Madison, WI). For the remainder of the study, the animals were housed in identical groupings as before in accordance with the university animal care and use committee regulations.

Immunization of cats with commercially available vaccines

The cats were randomly distributed into 5 groups (each n = 8). Each group received 1 of 4 commercially available vaccines according to the manufacturer's specifications and one group served as the unvaccinated control. Group A received the adjuvanted whole inactivated virus (WIV) vaccine Fel-O-Vax Lv-K® (Fort Dodge Animal Health, Overland Park, KS). Group B received FEVAXYN FeLV® (Schering-Plough Animal Health Corporation, Summit, NJ), also an adjuvanted WIV vaccine. Group C received the adjuvanted, inactivated mixed subunit vaccine LEUKOCELL 2® (Pfizer Animal Health, New York, NY). Group D received PROTEX®-FeLV (Intervet, Millsboro, DE). It was a non-adjuvanted WIV vaccine which is no longer commercially available. The priming vaccination was administered when the cats were 15 – 16 weeks

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of age. The boosting vaccination was administered 3 weeks later when the cats were 18 – 19 weeks of age.

In addition, a concurrent feline immunodeficiency virus (FIV) vaccine experiment was performed using the same animals. The FIV vaccine study will not be discussed further because it had no statistically significant effect on the outcome of the FeLV vaccine experiment.

FeLV-A/61E challenge

Four months after receiving their boosting immunization, at 34 – 35 weeks of age, all cats were challenged intraperitoneally with 200 μL of 5×10^4 $TCID_{50}$/mL FeLV-A/61E. This subgroup A virus strain is the highly replication competent, non-acutely pathogenic component of the FeLV-FAIDS complex (7, 30, 48, 51). The cell-free infectious virus inoculum was prepared as supernatant from AH927 feline fibroblast cell cultures and determined to be equivalent to 1 CID_{100} (100% cat infective dose). Cats were observed twice daily for signs of illness after virus inoculation.

Sample collection and processing

Sample collections were performed on cats anesthetized with an intramuscular administration of ketamine hydrochloride (11 mg/kg). Blood samples were collected at challenge and every week thereafter through 8 weeks post-challenge (PC). Whole blood was shipped overnight on ice to Colorado State University (Ft. Collins, CO) where it was immediately processed upon arrival. Buffy coat cell pellets were stored at -70°C until analysis for FeLV DNA by qPCR. Plasma samples were separated into 1 mL aliquots

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and stored at -70°C until analysis for FeLV RNA by qPCR, FeLV p27 capsid antigen by capture ELISA, and infectious FeLV. Sera samples were stored at -70°C until analysis for FeLV neutralizing antibody.

Detection of FeLV DNA and RNA by qPCR assays

A weekly analysis of buffy coat cells for viral DNA and plasma for viral RNA was achieved using a quantitative real-time PCR (qPCR) and reverse transcriptase qPCR, respectively. The DNA and RNA extractions, primer/probe set, and assay conditions were as described (71, 72). The assays amplify exogenous and not endogenous FeLV sequences within the U3 region of FeLV-A/61E (1, 2). The viral loads of the samples were determined by comparing the threshold cycle (C_T) value of the samples' nucleic acid with the standard curve of the co-amplified standard template. End-point dilutions of the standard templates yielded lower detection limits of 5 copies/DNA qPCR reaction and 10 copies/RNA qPCR reaction (1150 RNA copies/mL plasma).

Detection of FeLV by p27 capture ELISA

FeLV p27 capsid antigen was detected in plasma weekly, as previously described (72). In addition, sera were assessed by the SNAP® FeLV antigen diagnostic test (IDEXX Laboratories, Inc., Westbrook, ME). In rare instances of incongruous results, the perceived most sensitive finding of the two assays was chosen. In accord with USDA guidelines, a cat was considered FeLV-positive (infected) when 3 consecutive samples had positive results between weeks 3 to 8 PC (66).

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FeLV-A/61E stock

The FeLV-A/61E stock used for the virus infectivity and virus neutralization assays consisted of supernatant collected from Crandell feline kidney (CrFK) cells chronically infected with FeLV-A/61E. The virus stock was strongly positive by the p27 capsid ELISA and contained 4.4×10^9 copies/mL by RNA qPCR. The 50% endpoint dilution of the virus stock was 2.5×10^3 $TCID_{50}$/mL.

Detection of infectious FeLV

Plasma samples from cats inoculated with FeLV-A/61E 4 weeks previously were incubated with partially confluent AH927 cells and the presence of infectious FeLV (VI) was determined by p27 capsid antigen ELISA of the cell culture supernatant. Specifically, AH927 cells were seeded on a 24-well plate at a concentration of 1×10^5 cells/well. Cells were grown in 1 mL of minimal essential medium containing Earle's salts and L-glutamine (Gibco Products, Invitrogen Corp., Carlsbad, CA) with the addition of 1% penicillin/streptomycin, 10% inactivated fetal bovine serum, and 4 μg/mL polybrene (Sigma-Aldrich Corp., St. Louis, MO). Following an overnight incubation at 37°C with 5% CO_2, medium was removed from the subconfluent cells and 400 μL of freshly thawed sample plasma was added to each well. After a 2 hour incubation, the plasma was replaced with 1 mL of fresh medium without supplementation of polybrene and the plates were placed at 37°C with 5% CO_2. This was day 1. The cells were examined daily for confluency and any cytopathic effects and were passaged (1:4 cell split) as necessary, approximately every 3 days. At each passage, the supernatants were assessed, in duplicate, for FeLV p27 antigen by the capture ELISA. The infectivity assay

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was stopped at day 21 and if a mean ELISA absorbance value of 0.05 or more was obtained (a threshold set above the negative control plus three times the standard deviation), the plasma sample was considered positive for infectious FeLV. Sham inoculated wells served as the negative control and the FeLV-A/61E virus stock served as the positive control for the virus infectivity assay.

Detection of FeLV neutralizing antibodies

Virus neutralizing (VN) antibodies were measured at 2 time-points; post-vaccination just prior to receiving the virus challenge and at 8 week PC. Briefly, AH927 cells were seeded on a 96-well plate at a concentration of 1×10^4 cells/well and grown overnight in 100 μL of the same media and under the same conditions as that used in the VI assay. The following day (day 1), sample sera were heat inactivated for 30 minutes at 56°C and 2-fold serial dilutions made (1:2 to 1:256). An equal volume (50 μL) of each serum dilution and 10 $TCID_{50}$/mL FeLV-A/61E were incubated alone, in triplicate, for 1 hour at 37°C with 5% CO_2 to allow antibody binding. Medium was removed from the subconfluent AH927 cells and the serum/virus mixtures were transferred to the cells to allow residual infectivity. After a 2 hour incubation at 37°C, the serum/virus mixtures were removed, the cells were washed 3 times before being refed with 200 μL fresh media without polybrene, and the plates were placed at 37°C with 5% CO_2. On day 4, approximately 50% of the media was removed (100 μL) and the cells refed with 150 μL fresh media. The VN antibody assay was stopped on day 7 and cell culture supernatants from each well were assessed, singly, for FeLV p27 antigen by capture ELISA. If the mean optical density value of each triplicate serum dilution was less than 0.05, the serum

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sample was considered positive for VN antibody. The titer of antibody was taken as the reciprocal of the sera dilution. Naïve serum served as the negative control and FeLV regressor serum served as the positive control for VN antibodies, respectively. In addition, sham inoculated wells served as the negative control and a back-titration of the virus stock served as the positive control for virus infection, respectively.

Data analysis

Undetectable results by both qPCR assays were corrected to a value of one and then all viral RNA and DNA levels were log transformed. Statistically significant differences in viral DNA and RNA levels between the FeLV:host categories and between the experimental groups were determined using repeated-measure analysis of variance (ANOVA) with the Tukey-Kramer post-hoc test. The chi-square test was used to determine if VI assay results and the FeLV:host categories were independent. Kappa statistics for the DNA and RNA qPCR assays, the p27 capture ELISA, and the VI assay were calculated. Pearson correlation coefficients were determined to assess the linear relationship between viral DNA and RNA levels, p27 levels, and infectious virus levels. After a Fisher's r to z transformation, p values were obtained. A statistically significant difference between groups was considered to have occurred when a p value was < 0.05. Statistical tests were performed using StatView® version 5.0.1 for Macintosh, copyright 1999 (Abacus Concepts, Inc., Berkeley, CA).

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RESULTS

Reproducible host:virus relationships based on circulating p27 and viral DNA levels

We previously defined 4 categories of FeLV infection using circulating p27 and viral DNA levels (71). Here we examined weekly 40 FeLV-vaccinated and unvaccinated cats that were challenged with the same FeLV strain by a different route (intraperitoneal instead of oronasal) and found these categories to be reproducible. In 15 cats neither antigenemia nor viral DNA were ever detected in blood at any time-point. These animals were designated as having experienced abortive infection, now simply expressed as a Category 1 response. Five cats in which p27 antigen was never detected yet viral DNA was detectable in blood albeit in a transient low or persistent low mode (median: 176 copies/10^6 buffy coat cells; range: undetectable to 5,675 copies/10^6 buffy coat cells) were classified as having experienced regressive infection, expressed as Category 2. One additional cat without detectable antigen or viral DNA was classified into Category 2 based on detectable plasma viral RNA (see section below). Four cats classified as Category 3 (previously called latent infection) developed transient antigenemia and retained a low to moderate circulating viral DNA load (median: 2,487 copies/10^6 buffy coat cells; range: undetectable to 57,252 copies/10^6 buffy coat cells). Lastly, 15 animals that developed persistent antigenemia and persistent high circulating viral DNA (median: 934,891 copies/10^6 buffy coat cells; range: 57 to 4,173,516 copies/10^6 buffy coat cells) were classified as Category 4 (aka progressive infection). It is uncertain whether cats classified into Category 2 or 3 have non-productive viral states or antigen levels below assay sensitivity when circulating viral DNA is detected in the absence of antigenemia.

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Viral DNA initiates transcriptionally active infection

To determine if detectable viral DNA is transcriptionally active in the absence of antigenemia, we developed and validated a real-time reverse transcriptase qPCR assay to detect circulating viral RNA (72). We previously reported the almost perfect agreement and the extremely strong linear relationship between viral DNA and RNA levels (Table 3.1) (72). In agreement with this finding, the four host:virus relationships were reinforced when the weekly viral RNA levels were combined with viral DNA levels and viral antigen status (Fig. 3.1). In the 15 cats grouped into Category 1 viral RNA was never detectable in blood at any time-point. In 4 of the 6 cats classified into Category 2, low and/or transient levels of viral RNA were detected (median: undetectable; range: undetectable to 120,267 copies/mL plasma) with consistently detectable viral DNA. In the remaining 2 animals included in Category 2, we observed either transient low proviral burden with undetectable RNA and antigenemia or transient low viral RNA load with undetectable DNA and antigenemia. The 4 cats designated into Category 3 all developed a transient low to moderate circulating viral RNA burden (median: 124 copies/mL plasma; range: undetectable to 255,238 copies/mL plasma) in the presence of persistent viral DNA and transient antigenemia. Lastly, 15 cats classified into Category 4 had a persistent high circulating viral RNA load (median: 2,681,905 copies/mL plasma; range: undetectable to 161,904,762 copies/mL plasma) correlating with the persistent high viral DNA and p27 levels.

Statistically significant differences ($p < 0.01$) in viral DNA and RNA levels distinguished all FeLV categories with the exception of Category 2 vs. 3 (i.e. Category 1 vs. 2; Category 1 vs. 3; Category 1 vs. 4; Category 2 vs. 4; and Category 3 vs. 4). Thus,

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these results indicated that in most instances, viral DNA initiates a transcriptionally active infection that may be sustained in the absence of detectable antigenemia. However, whether detectable viral nucleic acids were indicative of replication competent intact virions being produced remained uncertain.

Table 3.1. Agreement and correlation between FeLV DNA qPCR, RNA qPCR, p27 antigen ELISA, and the VI assay

	RNA		p27		VI	
	kappa	r	kappa	r	kappa	r
DNA	0.82	0.939*	0.56	0.776*	0.45	0.746*
RNA			0.68	0.428*	0.64	0.771*
p27					0.76	0.816*

A kappa statistic of 0.20 or less indicates slight agreement, 0.21 to 0.40 indicates fair agreement, 0.41 to 0.60 indicates moderate agreement, 0.61 to 0.80 indicates substantial agreement, 0.81 or more indicates almost perfect agreement, and 1.0 indicates perfect agreement.
* $p < 0.0001$

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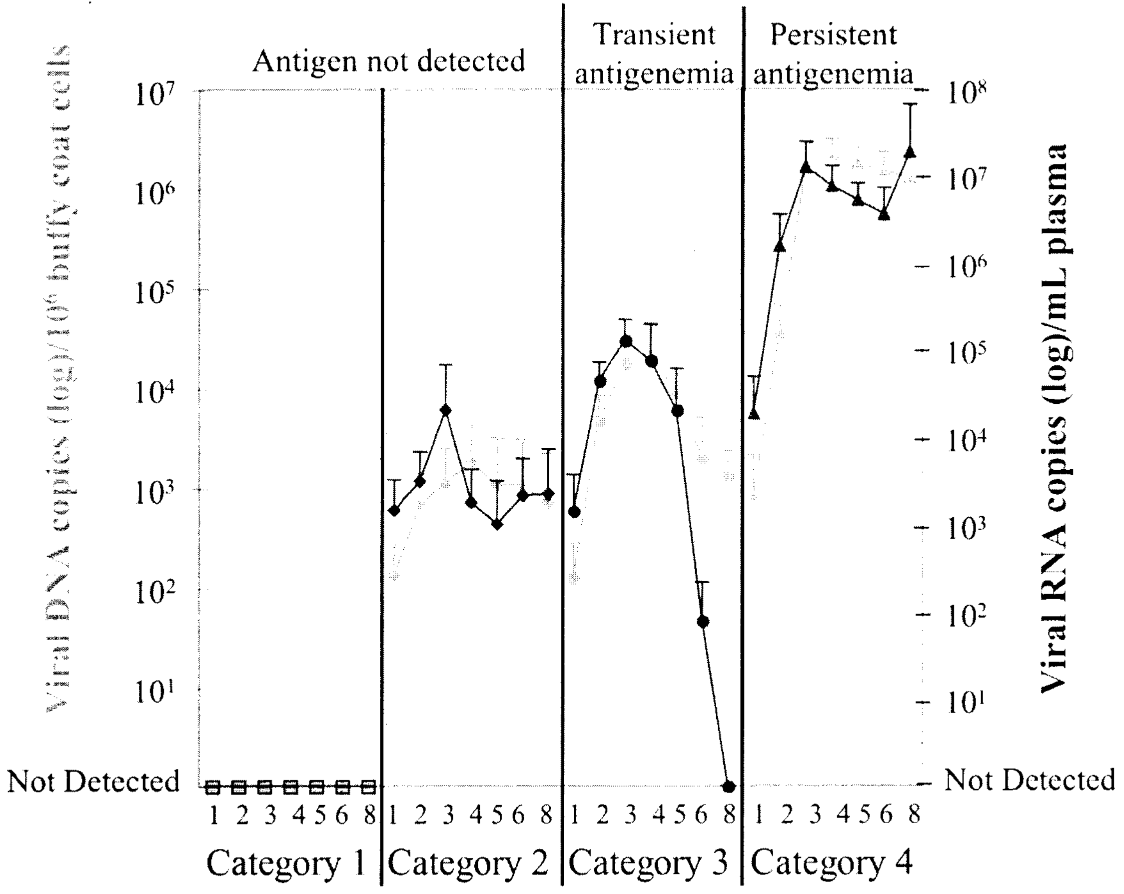

Fig. 3.1. Host:virus relationships emerged using circulating viral antigen in conjunction with correlative viral RNA and DNA levels. FeLV-A/61E-infected cats classified into Category 1 (squares) never had detectable antigen, viral RNA (black symbols), or viral DNA (gray symbols) in blood (n = 15). Cats grouped into Category 2 (diamonds) did not have circulating antigen detected but viral RNA and/or DNA was detectable in blood, albeit at transient low or persistent low levels (n = 6). Cats considered to be in Category 3 (circles) developed a transient antigenemia and viral RNA burden, yet retained low to moderate viral DNA levels in blood (n = 4). Cats in Category 4 (triangles) were persistently antigenemic and had persistent high circulating viral RNA and DNA levels (n = 15). Statistically significant differences ($p < 0.01$) in viral DNA and RNA levels were present between all FeLV categories (with the exception of Category 2 vs. Category 3): Category 1 vs. Category 2, Category 1 vs. Category 3, Category 1 vs. Category 4, Category 2 vs. Category 4, and Category 3 vs. Category 4. Mean ± SD are plotted.

High viral DNA and RNA levels are associated with infectious virus

To determine whether detectable viral RNA represented infectious virus in the absence of detectable antigenemia, plasma viral infectivity (VI) assays were performed at

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4 weeks PC. Infectious virus was detected only in 12 of the 15 cats classified into Category 4. In no sample was infectious virus detected without detectable viral DNA and RNA. In one Category 4 animal, infectious virus was detected yet antigenemia was not. In no sample in which viral DNA and RNA was undetectable (Category 1) was infectious virus detected. It is noteworthy that infectious virus was not detected in any cats with low to moderate viral DNA and RNA levels, when viral antigen was also undetectable (Category 2 and 3).

The chi square test confirmed an association between high viral DNA and RNA loads (Category 4) and ability to detect infectious virus ($p < 0.0001$), demonstrating that high levels of viral DNA and RNA ($>10^5$ DNA copies/10^6 buffy coat cells and $>10^5$ RNA copies/mL plasma) represent infectious virus. The significance of low to moderate viral DNA and RNA loads ($>10^1$ to $<10^5$ DNA copies/10^6 buffy coat cells and $>10^1$ to $<10^5$ RNA copies/mL plasma) remains undetermined. These levels may constitute either non-infectious virus or levels below assay sensitivities. Thus, viral DNA initiates a transcriptionally active infection, however, a productive viral state is only measurable when DNA and RNA levels are high.

Assay agreement and correlation

For each assay, the kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance (Table 3.1). An almost perfect agreement (kappa = 0.82) was indicated among the contemporary (DNA and RNA qPCR) assays and a substantial agreement (kappa = 0.76) was revealed among the traditional p27 capture ELISA and the VI assay. However, agreement among

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contemporary and more traditional assays (p27 and VI) was not as solid, with a more pronounced agreement between the RNA qPCR assay and the traditional assays (ELISA, kappa = 0.68; VI assay, kappa = 0.64) than between the DNA qPCR assay and the traditional assays (ELISA, kappa = 0.56; VI assay, kappa = 0.45).

The Pearson correlation coefficient was determined between viral DNA and RNA levels, p27 levels, and infectious virus levels (Table 3.1). Similar to the agreement observed between the contemporary and traditional assays, an extremely strong linear relationship was identified between viral RNA and DNA levels ($r = 0.939$, $p < 0.0001$) and a substantial linear relationship was present between p27 levels and infectious virus results ($r = 0.816$, $p < 0.0001$). Correlations among qPCR and traditional assays were mostly comparable: between DNA levels and p27 levels ($r = 0.776$, $p < 0.0001$); between DNA levels and infectious virus levels ($r = 0.746$, $p < 0.0001$); and between RNA levels and infectious virus levels ($r = 0.771$, $p < 0.001$). Surprisingly, the viral RNA load had a weak correlation with p27 levels ($r = 0.428$, $p < 0.0001$).

Two commercially available WIV FeLV vaccines induced 'sterilizing' immunity against FeLV infection

The FeLV:host relationship was also used to assess vaccine efficacy. Circulating viral DNA, RNA, and antigen levels were analyzed weekly and circulating infectious virus was assessed at 4 weeks PC.

In 7 of the 8 cats vaccinated with Vaccine A (Fort Dodge Animal Health Fel-O-Vax Lv-K®) neither viral DNA, RNA, antigenemia, nor infectious virus were detected (Table 3.2 and Fig. 3.2). In 1 of the 8 cats (#2), a persistent low proviral burden and a

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low transient viral RNA level were detected, however, neither antigenemia nor infectious virus were detectable. In 6 of 8 cats vaccinated with Vaccine B (Schering-Plough Animal Health FEVAXYN FeLV®) neither viral DNA, RNA, antigenemia, nor infectious virus were detected. One vaccinate (#9) had a transient low proviral burden without detectable RNA, antigenemia, or infectious virus, and the other recipient of Vaccine B (#11) had a persistent low proviral burden with a transient low viral RNA load but no detectable antigenemia or infectious virus. By contrast, in only 2 cats that received Vaccine C (Pfizer Animal Health LEUKOCELL 2®) were viral DNA, RNA, antigenemia, and infectious virus not detected. In three vaccinates (#21, #23, and #24), low to moderate levels of viral DNA and/or RNA were detected while antigen and infectious virus were not. The remaining 3 vaccinates had persistent high viral DNA and RNA levels, persistent antigenemia, and viremia. Finally, of the 8 cats receiving Vaccine D (Intervet PROTEX®-FeLV), 5 developed persistent high viral DNA and RNA loads, and persistent antigenemia; 4 of which had detectable infectious virus. In the remaining 3 vaccinates (#26, #31, and #32), viral DNA, RNA, and antigenemia were detectable PC but infectious virus was not. Of the unvaccinated control group, 7 of 8 cats developed persistent high viral DNA and RNA loads; 6 of these 7 cats developed persistent antigenemia, and in only 5 of the 7 was infectious virus detected at 4 week PC. The remaining unvaccinated cat (#40) had persistent low proviral and transient moderate viral RNA burdens with transient antigenemia and undetectable infectious virus.

Statistically significant differences (DNA and RNA $p < 0.01$) were detected between Vaccine A vs. unvaccinated controls and between Vaccine B vs. unvaccinated controls. Results for Vaccine C and Vaccine D were not statistically different from the

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unvaccinated control group. The preventable fraction (PF) is used to express vaccine efficacy due to the inherent resistance of some unvaccinated cats to development of persistent antigenemia after FeLV challenge (56). The PF is calculated as

$$\frac{\text{Incidence of Persistent Antigenemia in Controls} - \text{Incidence of Persistent Antigenemia in Vaccinates}}{\text{Incidence of Persistent Antigenemia in Controls}}$$

For vaccines A and B the PF was100%.

Our inability to detect any of several indicators of FeLV infection after administration of vaccines A and B appears to represent virtual 'sterilizing immunity' in the recipients.

Table 3.2. Efficacy of commercially available FeLV vaccines

Experimental group	n	Persistent antigenemia	Preventable fraction (%)
Vaccine A	8	0	100
Vaccine B	8	0	100
Vaccine C	8	3	50
Vaccine D	8	5	17
Unvaccinated controls	8	6	not applicable

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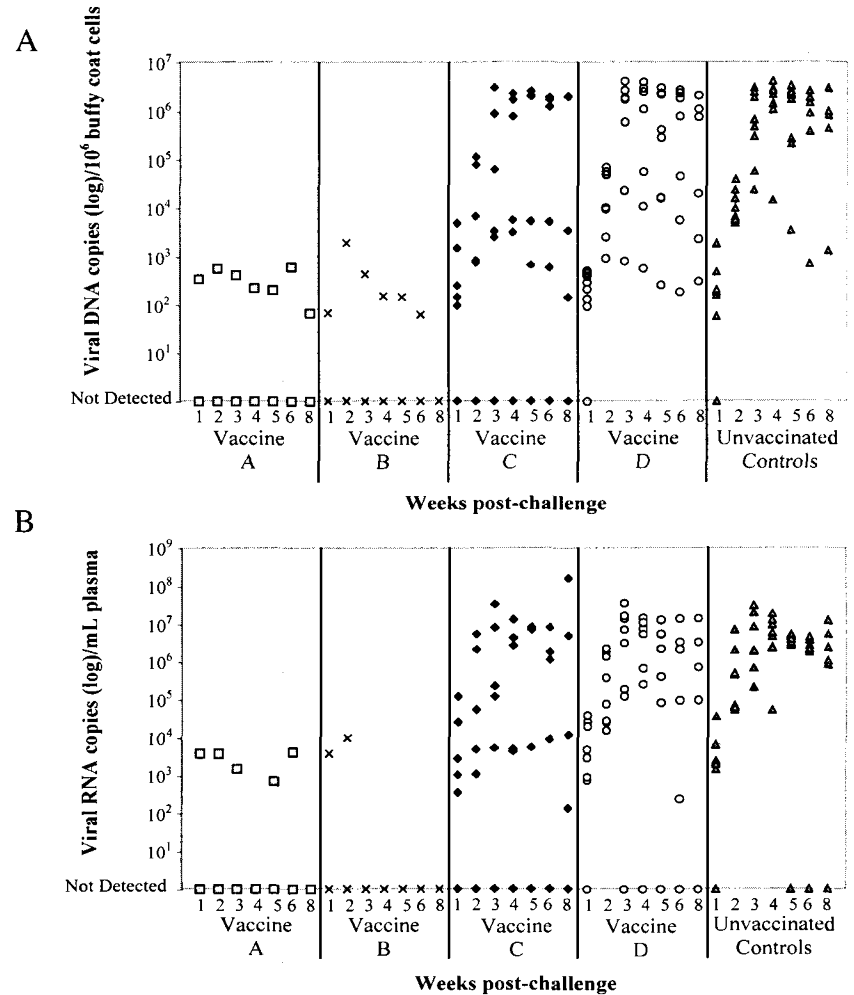

Fig. 3.2. Vaccine A (Fort Dodge Animal Health Fel-O-Vax Lv-K®) and Vaccine B (Schering-Plough Animal Health FEVAXYN FeLV®) induced sterilizing immunity against FeLV challenge. Seven of 8 cats vaccinated with Vaccine A (light gray squares) and 6 of 8 cats vaccinated with Vaccine B (black Xs) had undetectable viral DNA (A) and RNA (B). By contrast, only 2 cats which received Vaccine C (Pfizer Animal Health LEUKOCELL 2®, black diamonds) had undetectable viral DNA and RNA. All 8 cats which received Vaccine D (Intervet PROTEX®-FeLV, open circles) had detectable viral DNA and RNA PC. Seven of 8 unvaccinated control cats (dark gray triangles) had persistent high viral DNA and RNA levels. Statistically significant differences (DNA and RNA $p<0.01$) were detected between Vaccine A vs. unvaccinated controls and between Vaccine B vs. unvaccinated controls. Results for Vaccine C and Vaccine D were not statistically different from the unvaccinated control group.

Minimal virus neutralizing antibody in protected vaccinates

Virus neutralizing (VN) antibodies were measured post-vaccination just prior to receiving the virus challenge and at 8 week PC. FeLV vaccination was not distinguished

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by induction of substantial VN antibody titers (Fig. 3.3). Low VN antibody titers were observed in almost every vaccinated animal irrespective of the FeLV:host relationship that developed PC. Indeed, in all vaccinated cats in which viral DNA, RNA, antigen, or infectious virus were never detected, minimal VN immune responses were identified. In total, strong (1:16 to 1:128) VN antibody responses were detected in only 4 of 40 cats at 8 weeks post-challenge. All of these animals had eliminated circulating viral RNA more than 2 weeks earlier yet in all four, low viral DNA levels persisted in blood leukocytes. Thus, it appears that effective viral containment can occur despite a weak VN antibody response and in the presence of persistent low viral DNA levels.

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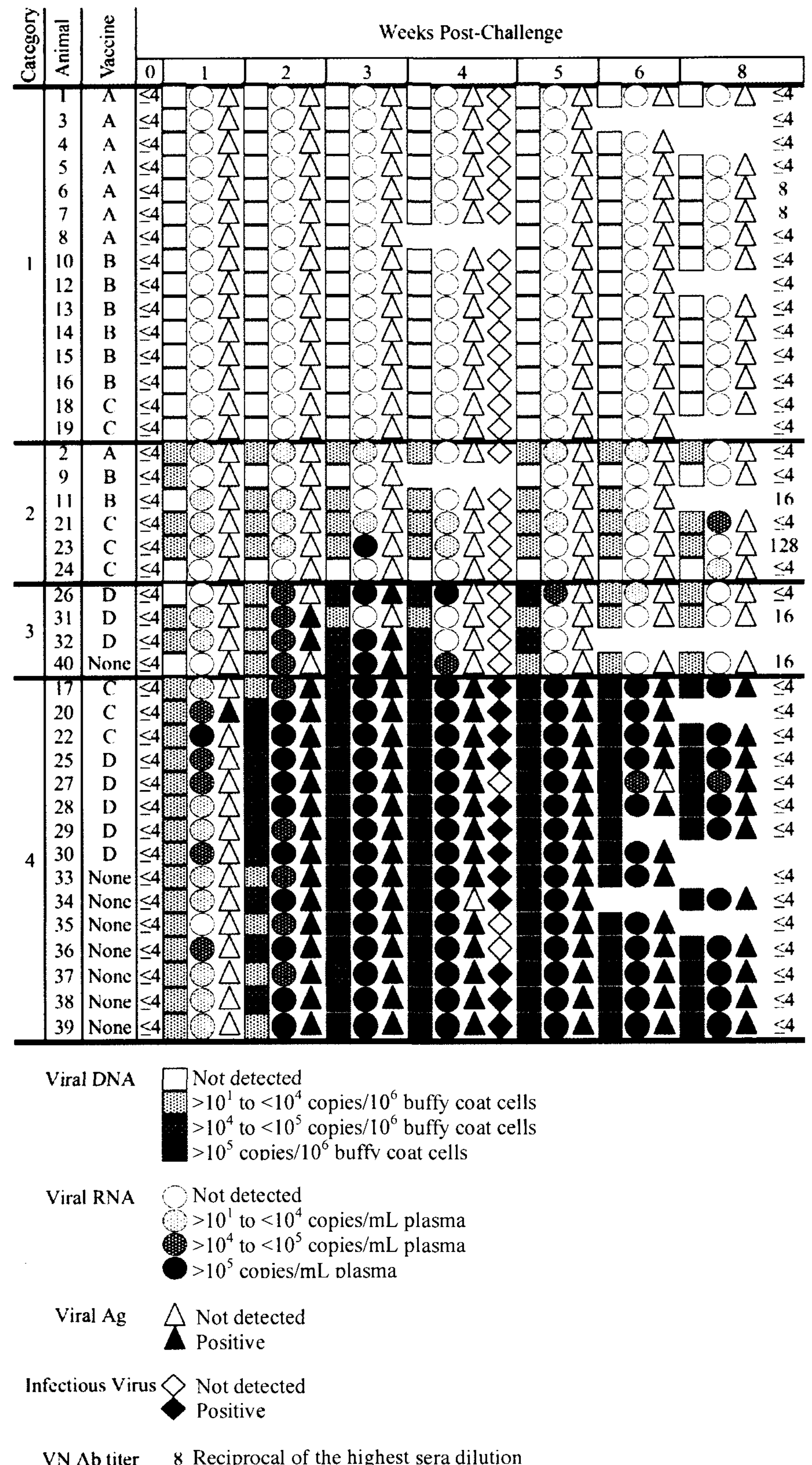

Fig. 3.3. Summary of FeLV:host relationships.

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DISCUSSION

Historically, FeLV has represented a diametric paradigm of effective host response leading to regressive infection vs. ineffective host response leading to progressive infection and disease (33). This model has been based on assays for viral infectivity (VI) (5, 8) or viral p27 Gag antigen either in blood cells by immunofluorescent antibody (IFA) assay (18, 19) or in plasma or serum by enzyme linked immunosorbant assay (ELISA) (43). Information obtained using these assays collectively indicated that in ~60% of cats exposed to FeLV, neither antigen nor infectious virus were detectable in the blood after virus challenge (16, 17, 27, 62). In stark contrast, ~30% of exposed animals developed persistent antigenemia and viremia. However, subsequent widespread use of the p27 ELISA, in combination with the IFA and VI assays, prompted the identification of cats with 'discordant' results (20, 35, 45, 46). In addition, several laboratories demonstrated that it is possible to reactivate FeLV from some cats with regressive infection (47, 58, 63). These observations introduced a more complex view of the FeLV:host relationship beyond the polar infection concept (27).

We and other investigators previously developed and applied quantitative real-time PCR (qPCR) assays to detect viral DNA, which demonstrated increased sensitivity vs. the p27 Gag ELISA (10, 25, 68, 71) and suggested a spectrum of more subtle host:virus relationships in antigen-negative cats (71). To determine whether the viral DNA detected was transcriptionally active, we developed a reverse transcriptase qPCR assay to quantitate extracellular FeLV RNA in plasma and compared these results with

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those for viral DNA, p27 capsid antigen capture ELISA, and viral infectivity assays in FeLV-vaccinated and unvaccinated cats challenged with FeLV-A/61E.

We found the DNA and RNA qPCR assays were in almost perfect agreement and had an extremely strong linear correlation (72), indicating that a substantial fraction of the detected FeLV DNA was integrated into the host cell genome and initiated a transcriptionally active infection. Similarly, we found the p27 ELISA and VI assays were in substantial agreement and positively correlated. Although antigenemia in most cases equated to viremia, we did identify a total of 4 'discordant' animals. We did not find this level of agreement however, between the qPCR and traditional assays. Most samples with low to moderate viral DNA and RNA loads did not have detectable antigen or infectious virus; however, p27 and infectious virus were easily detected when viral DNA and RNA loads were high ($>10^5$ DNA copies/10^6 buffy coat cells and $>10^5$ RNA copies/mL plasma). These results are in agreement with those of other investigators who found that viremia was detectable only in animals with high viral DNA and RNA burdens (10, 26, 68). The clinical significance of low to moderate viral DNA and RNA loads ($>10^1$ to $<10^5$ DNA copies/10^6 buffy coat cells and $>10^1$ to $<10^5$ RNA copies/mL plasma) in non-viremic, non-antigenemic cats remains undetermined.

We assume the measurement of extracellular (plasma) viral RNA indicates replication-competent intact virions nonetheless, it is possible that only a very small percentage of these circulating virions are actually infectious as has been observed in HIV infections (39, 55). Multiple factors could have contributed to our inability to detect infectious virus in animals with low nucleic acid levels, these include: sample deterioration, virus neutralizing antibody or some other inhibitory factor present in the

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inoculating plasma, the use of cell-free plasma inocula, virus dilution through cell culture passage during the VI assay, and the sensitivity of antigen capture ELISA as the read-out (6, 9, 41, 55, 64, 73). However, it seems most plausible that given the high sensitivity of RNA qPCR, low levels of infectious virus and antigen were produced but below the limits of detection. Consequently, the term 'latent' could be relative when describing animals which retain viral genome but do not produce detectable infectious virus particles.

The four putative FeLV:host response categories suggested by DNA qPCR were reinforced with the addition of circulating viral RNA levels. However, here we have reverted to numbered categories similar to those which have been offered by others in preceding reports (27, 44, 46), since terminology becomes to a degree awkward or confusing. Fifteen cats were classified into Category 1 (traditional regressors) because they did not have detectable antigen, viral DNA, or viral RNA in blood at any time-point. In this situation, these cats were indistinguishable from those never exposed to FeLV on the basis of viral DNA and RNA and antigen capture ELISA results. Statistically significant differences were observed between vaccinated and unvaccinated cats. Thus, our inability to detect indicators of virus infection was not due to lack of exposure instead, the vaccination primed the cats to resist infection. Of the 15 cats in Category 1, 7 received Vaccine A, 6 received Vaccine B, and 2 received Vaccine C. Consequently, it appears that Category 1 animals had effective host immune responses which abrogated infection and eliminated FeLV-infected cells within one week of viral exposure and that two of the four vaccines used were able to effectively prime this successful response.

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Our viral DNA and RNA qPCR assays have lower detection limits of 5 copies per DNA qPCR reaction and 10 copies/RNA qPCR reaction (1150 RNA copies/mL plasma), respectively. This level of qPCR sensitivity is similar to that of Tandon *et al.* who reported 1 copy per DNA qPCR reaction detected 38% of the time and 180 copies/RNA qPCR reaction (2250 copies RNA/mL) (68). It is probable however, that virus could have been detected at time-points earlier than 1 week PC. While it is possible that virus could have been harbored in some tissue, we previously did not observe this phenomenon (71). Our earlier report evaluated cats within each host:virus relationship, including those with undetectable viral DNA in the periphery, for viral DNA within multiple tissues (bone marrow, spleen, mesenteric lymph node, thymus, tonsil, and retropharyngeal lymph node) and we were unable to demonstrate any such viral reservoir. In fact, we found circulating viral DNA burdens were positively correlated with tissue viral DNA loads, in accord with others using titrated virus for the VI assay and semiquantitative PCR (32, 59, 60). Although descriptions of 'localized' or 'sequestered' infections exist, the foundation of these case reports was the sensitivity of conventional assays. We can now appreciate how these animals had sufficient virus in plasma (22) or milk (52) to transmit to others despite having no or intermittently detectable antigenemia, respectively.

Six cats were classified into Category 2 on the basis of the continual absence of detectable antigen but the presence of viral RNA and/or DNA detectable in blood. In the typical course of Category 2 infection, FeLV-infected cells (buffy coat provirus positive) launched a transcriptionally active infection (plasma viral RNA positive) by the first week post-challenge (PC). In half of these animals, the provirus became quiescent

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(plasma viral RNA negative) within the first 8 weeks PC despite the persistence of infected cells (in one animal, DNA-bearing cells were also eliminated). In one animal, we detected viral RNA one sampling time-point before viral DNA. This phenomenon, also observed by Hofmann-Lehmann (26), may represent the detection of a primary cell-free viremia in which virus is replicating in local lymphoid tissue but viral DNA containing cells are not yet circulating, or perhaps we detected the original challenge virus inoculated intraperitoneally. Similar to cats in Category 1, we believe that relatively effective viral containment is reflected by vaccination in that all cats in Category 2 received vaccination (1 in Vaccine A group, 2 in Vaccine B group, and 3 in Vaccine C group). That this FeLV:host relationship is potentially dynamic making viral escape possible was suggested by 2 cats with increased viral RNA levels at 8 weeks PC.

Four cats were grouped into Category 3 by the presence of antigenemia, albeit transiently, with concurrent detection of circulating viral RNA and DNA. Unlike Category 2, Category 3 appeared to be less variable as all animals had a similar course of infection. Upon virus challenge, it appeared that viral DNA was quickly integrated into the host cells' genomes and promptly transcribed as evidenced by the simultaneous detection of viral DNA and RNA. Viral nucleic acid levels increased rapidly to peak at ~3 weeks PC, and at this peak plasma p27 was detectable. Transcription was then dampened and eventually silenced by week 8 PC. Although all 4 cats cleared circulating viral RNA, they retained infected cells in the periphery. These FeLV:host relationships became established by 8 weeks PC as with our previous findings (71). While viral reactivation is possible we, and others, have observed that these relationships are maintained for years and a recurrence of antigenemia is a rare event (24, 71). Pepin and

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co-workers (54) recently examined viral DNA and RNA loads within leukocyte subsets of provirus-positive/antigen-negative animals and found that years after FeLV exposure viral DNA was carried primarily in CD4+ T cells and B cells. Moreover, viral DNA was not found in granulocytes, giving compelling evidence for a primary lymphocyte/monocyte-associated viremia with viral containment occurring before bone marrow involvement and the subsequent secondary granulocyte-associated viremia as originally posited by Rojko *et al.* (62). FeLV transmission from non-antigenemic and intermittently antigenemic cats has been reported (22, 52), a possibility not assessed in the discordant cats (detectable nucleic acids without detectable viremia or antigenemia) in the present study. Such issues are pertinent to use of FeLV antigen-negative cats for blood donation, tissue transplants, and adoptions, as well as to the use of therapeutic immunosuppressive drugs in antigen-negative cats (4, 13, 49, 50). Until the clinical significance of nucleic acid-positive/antigen-negative animals is understood, the diagnostic significance of applying FeLV qPCR in client-owned cats remains unclear.

Finally, 15 cats were designated into Category 4 (traditional progressors) as these animals had persistent high viral DNA, RNA and antigenemia. By the first week PC, viral DNA was transcribed in all but 1 cat, although antigenemia was not detected in all animals until 2 weeks PC. Similar to Category 3, peak viral DNA and RNA loads were reached in most cats by 3 weeks PC. These high levels were maintained throughout the rest of the study in all but one animal in which RNA and DNA levels dropped slightly. Despite having similar proviral loads and slightly higher viral RNA levels compared to Category 2 and 3 at the 1 week PC time-point, Category 4 animals failed to restrict viral replication. Research by Quackenbush and co-workers (59, 60) found the highest viral

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DNA burden in B lymphocytes of long-term persistently infected animals but provirus was also detected in CD4+ and CD8+ T lymphocytes using semi-quantitative PCR. When leukocyte subsets were analyzed for intracellular p27 capsid antigen, higher percentages of monocytes and granulocytes were identified as expressing p27 than lymphocytes (59, 60). Recently, Pepin *et al.* (54) used qPCR to evaluate long-term persistently infected animals and found proviral loads uniformly high in all leukocyte subsets. But in agreement with Quackenbush *et al.* (59, 60), monocytes and granulocytes were found to have the most productive infections, as determined by higher viral RNA loads, compared to lymphocytes (54). This is additional support for ineffective viral containment leading to a secondary viremia in cells of bone marrow origin (62). Further examination of leukocyte subsets during the acute phase of FeLV infection, when proviral and viral loads are seemingly comparable, may help identify the cellular dynamics that influence the FeLV:host relationship during this pivotal period.

One of the objectives of this study was to employ qPCR along with the traditional FeLV assays to evaluate the efficacy of four FeLV vaccines that were commercially available (in the USA). Two whole inactivated virus (WIV) adjuvanted FeLV vaccines (Fel-O-Vax Lv-K® by Fort Dodge Animal Health and FEVAXYN FeLV® by Schering-Plough Animal Health Corporation) provided substantial protection against FeLV challenge. In nearly every recipient of these vaccines, neither viral DNA, RNA, antigen, nor infectious virus could be detected in blood. Thus, we found the preventable fraction for both vaccines was 100%--an impressive finding. Our results are consistent with previously reported preventable fractions (PF) of 86 – 100% for Fel-O-Vax Lv-K® (28, 29, 40, 71), PF of 90 – 100% for FEVAXYN FeLV® (23, 53), as well as PF of 35 – 88%

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for the Pfizer Animal Health vaccine LEUKOCELL 2® (14, 15, 40, 57, 70). In addition, several of these aforementioned studies attempted to isolate latent virus in bone marrow of non-antigenemic/non-viremic animals at terminal time-points without success (23, 28, 29, 40, 53). Although this was not specifically addressed in this study, we previously could not detect latent virus (viral DNA) in tissues, including bone marrow, of recipients of Fel-O-Vax Lv-K® negative for circulating viral DNA and antigen (71). Consequently, two of the WIV vaccines appeared effective for prevention of not only active but also latent infections. Our inability to detect evidence of viral infection by any assay employed at any point post-exposure in the vaccinated cats appears to represent virtual 'sterilizing immunity' in the protected vaccinates. These results lend support to the tenet that successful immunity to retroviral infection can be obtained with WIV immunoprophylaxis.

Similar studies using FeLV qPCR to evaluate vaccine efficacy have not identified sterilizing immunity (24, 26). While the qPCR assays used here and that of Tandon *et al.* (68) appear to have similar sensitivities, several other factors such as challenge virus strain, dose, route, age of vaccinates, and host genetics could all account for this discrepancy. The FeLV-61E subgroup A virus isolate used in the present study is a molecular clone of the highly replication competent, non-acutely pathogenic component of the FeLV-FAIDS complex (7, 30, 48, 51) whereas Hofmann-Lehmann *et al.* (24, 26) have used the moderately pathogenic FeLV-A/Glasgow-1. However, the two isolates are subtype A homologous. The virus challenge dosages we have used were satisfactory according to USDA guidelines (66) in that at least 60% of the controls developed persistent viremia--75% in the present study and 70% in a previous study (71). In the

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studies of Hofmann-Lehmann (24, 26) persistent viremia was induced in a higher percentage (83 – 90%) of unvaccinated controls consequently, a more potent challenge dose may have negated a partially effective immune response. Due to the inherent age-related resistance to FeLV (31), the age of vaccinates at challenge could also explain this difference. Not surprisingly, host genetics play a role in viral outcome and specifically the endogenous FeLV (enFeLV) load may influence the course of exogenous FeLV infection (69).

The final objective of our study was to assess the level of virus neutralizing (VN) antibody produced following vaccination and again 8 weeks PC as a means to identify immune correlates of protection. VN antibodies were not induced following vaccination and in only a few animals effecting successful viral containment were relatively low level VN antibody responses elicited. In animals with the highest VN antibody responses, viral DNA remained detectable in circulating cells. Our observations may be due to a limited sensitivity of our VN antibody assay yet, we did detect low levels of FeLV neutralizing activity (1:4) in some unvaccinated cats prior to FeLV exposure. There is precedent for such low level gammaretrovirus neutralizing activity to be associated with a soluble non-immunoglobulin, non-complement inhibitor (9, 41, 73). Resistance to FeLV infection has been suggested to be associated with protective levels of VN antibodies in non-viremic animals (14, 17, 26, 31, 34, 36, 47, 52, 65) although responses have been modest and less than consistent. Moreover, previous FeLV vaccine studies have observed that resistance to infection was attained despite the absence of a detectable VN antibody response (15, 21, 53). This lack of association between humoral responses and effective viral containment suggests a role for protective cell-mediated immune

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responses. Indeed, Flynn *et al.* (10, 11) have demonstrated an association between modest levels of FeLV-specific cytotoxic T lymphocyte activity in cats that resisted infection. Yet in summary, despite a highly effective vaccination the determinants of effective FeLV immunity remain far from understood.

In conclusion, we have used contemporary methodologies increasing the sensitivity of FeLV detection to study innate and vaccine-primed FeLV resistance. The results help us to understand instances of discordant results using traditional assays and offer opportunities for further insight into FeLV:host relationships. In particular, further long-term studies are needed to determine the clinical relevance of detection of viral nucleic acids in non-antigenemic/non-viremic cats. Also merited is additional focus on the FeLV infection dynamics in very early infection, when virus containment or lack thereof transpires. Finally, we have presented evidence for virtual sterilizing immunity against a retroviral infection elicited by WIV vaccination. Further examination of the cell-mediated immune response is a requisite to unraveling the immune correlates of protection. The above findings reinforce the precept of FeLV infection as a model of the early immune responses that determine effective vs. ineffective retroviral containment, offering valuable insights into immunoprevention and therapy.

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CONCLUSION AND FUTURE DIRECTIONS

The research described in this dissertation offers new insights into the FeLV:host relationship. Contemporary PCR methodologies allowed detection of FeLV DNA in circulating cells and FeLV RNA in plasma of non-antigenemic/non-viremic cats that had ostensibly resisted viral challenge. Because almost perfect agreement was demonstrated between viral DNA and RNA levels, we infer that detected FeLV DNA is both integrated and transcriptionally active. Thus, we revealed a spectrum of virus:host relationships in cats exposed to FeLV: 1) undetectable antigenemia, viral DNA, and viral RNA; 2) undetectable antigenemia but low viral DNA and RNA levels; 3) transient antigenemia and moderate viral DNA and RNA levels; and 4) persistent antigenemia and high viral DNA and RNA levels. These host:virus relationships were established by 8 weeks post-challenge and maintained for years. Because viral DNA levels in circulation and tissues was highly correlated, we surmise that exposed cats in which viral DNA was not detectable in blood cells did not maintain a tissue reservoir.

A reassessment of the FeLV:host relationship is not without consequence for client-owned cats. The clinical relevance of detection of viral nucleic acids in non-antigenemic/non-viremic cats is unknown. Further long-term studies are needed to determine the transmissibility by such animals and their risk for development of

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FeLV-related disease. A retrospective examination of blood or tumor cells for viral DNA and plasma for viral RNA in non-antigenemic animals with clinical suspicion of FeLV-related disease offers a noninvasive alternative. In addition, 4 distinct categories of FeLV:host relationships provide an ideal model to investigate determinants of effective viral containment. To determine in which phase of FeLV infection the virus has overcome the host immune response (or the host immune response has overcome the virus), we could examine circulating cells and tissue for viral DNA during the very early (days) post-challenge. In addition, we could examine proviral loads in leukocyte subsets during these critical first days post-challenge. To investigate the potential for viral factors which influence the FeLV:host relationship, we could clone and sequence segments of the genome most likely responsible for virulence (the region of *env* encoding the surface glycoprotein gp70).

These studies also demonstrated that two whole inactivated virus (WIV) adjuvanted FeLV vaccines provided very effective protection against FeLV challenge. In nearly every recipient of these vaccines, neither viral DNA, RNA, antigen, nor infectious virus could be detected in blood. Moreover, effective viral containment occurred despite a weak virus neutralizing antibody response. This lack of association between humoral responses and effective viral containment suggests a role for protective cell-mediated immune responses. To determine possible early cell-mediated immune correlates of protection, we could examine virus-specific circulating and lymphoid tissue type 1 and type 2 cytokine transcripts by mRNA qPCR and perform cytotoxic T cell assays.

The studies of FeLV:host relationships comprising this dissertation have contributed to understanding instances of discordant results using traditional assays. The

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above findings also reinforce the precept of FeLV infection as a model of the early immune responses that determine effective vs. ineffective retroviral containment. There remain many important questions to be addressed in the FeLV research field. Knowledge obtained from further studies will provide valuable insights into the genesis of effective anti-retroviral immunity.

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