Determination of Feline Leukemia Proviral Sequences in Naturally Infected Cats

Courtney Gorrell

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Determination of Feline Leukemia Proviral Sequences in Naturally Infected Cats

Abstract
Felines are susceptible to many different viruses throughout their lifetime, one being Feline Leukemia Virus (FeLV). FeLV is a transmittable RNA retrovirus that inhabits the cells of feline species' immune systems leading to possible infections and diseases such as seizures and other neurological disorders, anemia, stomatitis, enteritis, and lymphoma. There are four different types of this virus, FeLV-A, FeLV-B, FeLV-C, or FeLV-T, that enter cells via different cellular receptors causing different symptoms. FeLV-A is horizontally transmittable, and all FeLV-positive cats carry this form. Of the subtypes, FeLV-B is the most common, being found in approximately 50% of all cases, most often in cats with tumors and other abnormal tissue growths. The aim of this research was to determine the proviral sequences of FeLV-B in multiple tissue samples collected from a FeLV positive cat. Genomic DNA from multiple tissue samples of a naturally infected cat with FeLV-B was extracted and digested with a set of restriction enzymes. Adapters were ligated to known sequences using genome walking and PCR was used to amplify fragments containing proviral FeLV. Samples were sent for sequencing, and the results used to design new primers for further work. It was hypothesized that of the six tissue samples, the tumor samples would show mutations and/or rearrangements not observed in other tissues. FeLV-B was confirmed in five of the six tissue samples extracted. When comparing known FeLV-A and -B sequences, the samples tissues were more closely related to the known FeLV-B. Variations in the tumor samples were observed in the nucleotide sequences and when these variations were altered, mutations within the amino acid sequences were observed.

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Keywords
FeLV-B, tumor tissues, sequence alignment, DNA sequences

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DETERMINATION OF FELINE LEUKEMIA PROVIRAL SEQUENCES IN NATURALLY INFECTED CATS
By
Courtney Gorrell
A Senior Thesis Submitted to the
Eastern Michigan University
Honors College
in Partial Fulfillment of the Requirements for Graduation
with Honors in Chemistry

Approved at Ypsilanti, Michigan, on this date April 15, 2016
Abstract

Felines are susceptible to many different viruses throughout their lifetime, one being Feline Leukemia Virus (FeLV). FeLV is a transmittable RNA retrovirus that inhabits the cells of feline species' immune systems leading to possible infections and diseases such as seizures and other neurological disorders, anemia, stomatitis, enteritis, and lymphoma. There are four different types of this virus, FeLV-A, FeLV-B, FeLV-C, or FeLV-T, that enter cells via different cellular receptors causing different symptoms. FeLV-A is horizontally transmittable, and all FeLV-positive cats carry this form. Of the subtypes, FeLV-B is the most common, being found in approximately 50% of all cases, most often in cats with tumors and other abnormal tissue growths. The aim of this research was to determine the proviral sequences of FeLV-B in multiple tissue samples collected from a FeLV positive cat. Genomic DNA from multiple tissue samples of a naturally infected cat with FeLV-B was extracted and digested with a set of restriction enzymes. Adapters were ligated to known sequences using genome walking and PCR was used to amplify fragments containing proviral FeLV. Samples were sent for sequencing, and the results used to design new primers for further work. It was hypothesized that of the six tissue samples, the tumor samples would show mutations and/or rearrangements not observed in other tissues. FeLV-B was confirmed in five of the six tissue samples extracted. When comparing known FeLV-A and -B sequences, the samples tissues were more closely related to the known FeLV-B. Variations in the tumor samples were observed in the nucleotide sequences and when these variations were altered, mutations within the amino acid sequences were observed.
Acknowledgments

I would first like to thank Dr. Heather Holmes, my research advisor. Had it not been for her dedicated involvement in my research as well as our team’s, this senior thesis would not have been possible. Thank you for hard work, understanding, and support for the past two years. I would also like to recognize and show appreciation to fellow researchers Alexa Salsbury, Hosam Issa, Jasmine Winzeler, Andre Tackett, and Brianna Sohl. We have all learned from each other’s work which has allowed us further understand research results. Additionally, I would like to thank Eastern Michigan University’s Chemistry Department as well as the Honors College for their continuous educational and financial support. Finally, the Country Cat Clinic and Leuk’s Landing Leukemia Rescue for providing FeLV-positive cat tissue samples for testing.
Table of Contents

Abstract ........................................................................................................................................ ii
Acknowledgments ...................................................................................................................... iii
Table of Contents ....................................................................................................................... iv
Introduction.................................................................................................................................. 1

Figure 1: Integrated FeLV proviral DNA. The double-stranded DNA provirus is integrated
into the host genome after reverse transcription of viral DNA. ............................................ 2

Figure 2: FeLV Structure. FeLV is a gamma-retrovirus measuring 110 millimicrons in
diameter. The envelope consists of surface and transmembrane proteins, which allows the
virus access to its host cell. ........................................................................................................ 3

Table 1: FeLV Subtypes. The cell entry methods, symptoms, and unique features of FeLV
subtypes compared to clarify differences between each subtype. .......................................... 5

Methods and Materials .......................................................................................................... 5

Extraction of Genomic DNA from Tissue Samples .................................................................. 5

Preparation of Tissue Lysates ................................................................................................... 6

Binding, Washing, and Eluting DNA from Tissue Lysates ....................................................... 6

Polymerase Chain Reaction Primers ......................................................................................... 7

Table 2: PCR Primers. Primers used in primary PCR. ............................................................... 7

Polymerase Chain Reaction ....................................................................................................... 7

Table 3: Conditions used to Primary and Secondary PCR processes ..................................... 7

Table 4: PCR conditions used for thermocycler ...................................................................... 7

Purification of PCR Amplicons .................................................................................................. 8

Digestion of DNA for Genome Walking Libraries ................................................................. 8

Post-Digestion Purification ....................................................................................................... 9

Ligation of Adapter Sequences to Purified DNA Digests ........................................................ 9

Gel Electrophoresis .................................................................................................................. 9

Results and Discussion ............................................................................................................ 10

Figure 3: Primary PCR from tissue samples. To verify the presence of FeLV in the extracted
tissue samples, Primary PCR was done using primer B5F/B5R. Lanes are as followed: 1-
Spleen, 2- Liver, 3-Mammary Tumor, 4- Reference ladders, 5- Lymph, 6- Kidney, 7- Brain.

Figure 4: Sequencing Alignment. These images compare sequences obtained from extracted DNA from tumor sample. The sequences are aligned with known FeLV-A sequence, Rickard-A, and FeLV-B Glasgow sequence-B, K01209. The known sequences are imputed as Query and the sample DNA is the subject. These results show that our sample is more closely correlated with FeLV-B than it is to FeLV-A.

Figure 5: Chromatogram Sequence. Sequencing chromatogram for the amplicon of FeLV-B obtained from genomic DNA extracted from mammary tumor tissue sample.

Figure 6: ClustalX alignment of tissue samples. Both native and reverse complement of the tissue samples were aligned together. See Table 5 for tissue sample with corresponding reference numbers.

Table 5: Sequence reference numbers with corresponding tissue samples.

Figure 7: ClustalX alignment of tissue samples. FeLV-A and FeLV-B reference sequences aligned with edited tumor sequences and other tissue sample sequences.

Figure 8: Alignment of amino acids. Alignment of deduced amino acids from the tumor DNA sequences shown in Figure 7. (a) Prolines resulting from a silent mutation of the DNA. (b)

Conclusion and Future Directions

References
Introduction

Feline leukemia virus is a simple gamma retrovirus that impacts the health of both domestic and wild cat populations. This retrovirus is horizontally transmitted through direct contact; frequently contaminated saliva from viraemic cats is transferred into the oropharynx of susceptible cats. With horizontal transmission from cat to cat, it has been estimated that nearly half of the urban domestic cat population will become infected with FeLV during a lifetime.[1] Once the virus integrates itself into the host’s DNA, it remains there until the host passes away. Due to this characteristic, the virus can be problematic for large cat species such as Puma concolor coryi, the Florida puma. This species became endangered in the late 1990s and with the identification of FeLV antigen-positive pumas during the capture season in 2002-2003, an investigation was launched to observe the progression of FeLV in the species. It was found that between January 1990 and April 2007, there was an increase in the proportion of pumas testing antibody-positive for FeLV. Vaccination was used to try to treat the epizootic and although there have been no new reported cases since July 2004, there is still a chance the virus could be reintroduced to the species.[2]

In 1964, William Jarrett discovered feline leukemia virus, which was the first identified feline retrovirus.[3] Similar to the retrovirus of rodents, gibbons and koalas, FeLV is a simple gamma retrovirus.[4] Although there are seven types of retroviruses, they all contain similar genomic structures. The genome consists of three mutual genes, pol, gag, and env, that sit between the upstream and downstream long terminal repeats (LTRs). The pol gene encodes those enzymes that are needed for replication and integration, such as reverse transcriptase and integrase. The gag gene encodes the main proteins of the virion, and the env gene encodes the glycoproteins of the envelope, the surface glycoproteins (SU) and the transmembrane protein
Variations in the SU affect receptor selection and binding affinity which then influence the rate of tissue tropism, virus spread and disease variety. The inner core of FeLV is made from p27, the capsid protein, which is enclosed by two envelope glycoproteins, the surface gp70, and the transmembrane protein p15E.

Figure 1 shows the structure of proviral FeLV. The LTR of FeLV in natural isolates shows significant genetic variation and these variations in LTRs have been associated with certain disease outcomes. For example, FeLV proviruses typically contained only a single copy of the enhancer in LTRs when obtained from non-T-cell malignancies. But for the FeLV proviruses cloned directly from thymic lymphomas of T-cell origin, LTRs contain two or three tandemly repeated enhancers.

![Figure 1: Integrated FeLV proviral DNA. The double-stranded DNA provirus is integrated into the host genome after reverse transcription of viral DNA.](image)

While feline retroviruses contain similar genomic structure, there are multiple types that help distinguish them, such as exogenous and endogenous viruses. An exogenous virus is one that is transmitted horizontally. Unlike an exogenous virus that is caused by an agent outside the host, an endogenous virus is one that is transmitted in the germ-line DNA of an infected host to another. Endogenous viruses are present in germ-lines due to residuals of ancient infections of animals by retroviruses. One type of the endogenous viruses in cats includes those endogenous FeLV-like genomes that have at least two families. These genomes contain many stop codons...
and are defective. They are never found as infectious viruses even though they, at times, express proteins such as envelope glycoproteins. The second endogenous virus, RD114, is not defective and while it may be expressed in cats, it rarely spreads \textit{in vivo} or \textit{in vitro} due to the lack of receptors for the virus in most cat cells.\textsuperscript{[3]}

During the first stage of the viral replicative cycle, an RNA genome with encoded genetic information of the retrovirus is copied into DNA assisted by reverse transcriptase. This enzyme disrupts the typical cellular flow of genetic information from DNA to RNA to protein. The DNA copy of the RNA genome is synthesized and then incorporated into the target cell’s genome as a provirus. Integrase, a viral enzyme, aids in this process. For the lifespan of the infected cell, the provirus persists in the genomic DNA, providing a template for viral gene expression, leading to progeny virions and virus shedding.\textsuperscript{[4]}

\textbf{Figure 2: FeLV Structure.} FeLV is a gamma-retrovirus measuring 110 millimicrons in diameter. The envelope consists of surface and transmembrane proteins, which allows the virus access to its host cell.
FeLV does not carry genetic information that could code for its disease potential; it only encodes the genes needed for its structure and replication. Despite the simplicity of the FeLV genome, it actually occurs as a complex family of closely related viruses rather than a single genetic species. This is due to genetic variation that takes place during replication in vivo through error-prone reverse transcription as well as recombination with endogenous FeLV-related sequences in the cat genome, leading to a virus population that is genetically diverse. Changes also occur by rearrangements or deletions during proviral DNA integration into the host cell, and through viral transduction, when the host genome is obtained by the virus and it is copied into other host cells in conjunction with the viral genome. The effect of these changes has led to three sub-types of feline leukemia that can arise in cats already infected with the virus, FeLV-A, including FeLV-B, -C, and -T.[5]

Feline leukemia virus is separated into four distinct subtypes: A, B, C, and T. Due to the differences in cellular receptors used to enter the host, different symptoms arise from each strain. Any cat diagnosed with feline leukemia virus carries FeLV-A, which is responsible for interhost transmission and is the only one to be horizontally transmitted. FeLV-B is proposed to be a recombination of FeLV-A with endogenous FeLV-related env sequences and is found in approximately 50% of FeLV positive cats that are otherwise healthy. Isolates of FeLV-C are rare and only occur with FeLV-A or the combination of FeLV-A and FeLV-B. FeLV-T is a T-cell-tropic, cytopathic virus that has been isolated from nonvirulent forms of FeLV-A. Table 1 below shows cell entry methods, symptoms, and unique features of each subtype for better clarification of the differences.[8]
Table 1: FeLV Subtypes. The cell entry methods, symptoms, and unique features of FeLV subtypes compared to clarify differences between each subtype.\[^{[9,10]}\]

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Cell Entry Methods</th>
<th>Symptoms</th>
<th>Unique Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeLV-A</td>
<td>Thiamine transport protein receptor, THTR1</td>
<td>Hematopoietic, neoplasia</td>
<td>FeLV-A cells remain susceptible to FeLV-B, so vaccinations with this strain prevents infection by all 3 subtypes</td>
</tr>
<tr>
<td>FeLV-B</td>
<td>Inorganic phosphate transporter PiT1 and PiT2</td>
<td>Neoplastic diseases</td>
<td>Non-pathogenic on its own, only when recombined with FeLV-A</td>
</tr>
<tr>
<td>FeLV-C</td>
<td>Heme exporting protein receptor, FLVCR1</td>
<td>Severe anemia, leukemia</td>
<td>Developed anemia is typically fatal within 1 month</td>
</tr>
<tr>
<td>FeLV-T</td>
<td>PiT1 and co-receptor FeLIX</td>
<td>Fever, diarrhea, neutropenia, lymphopenia</td>
<td>Infects T lymphocytes leading to severe immunosuppression</td>
</tr>
</tbody>
</table>

For this study, tissue samples from necropsy of a cat diagnosed with FeLV-A and FeLV-B from Leuk’s Landing Leukemia Rescue were used. The focus of the study was to verify the presence of FeLV-B in the tissue samples obtained and to determine unique mutations present in the variable regions of the envelope and long terminal repeats. Previous studies have shown that these unique sequences can significantly alter disease progression. By identifying previously unknown variations, new information about FeLV can be exposed leading to better understanding of the virus.

Methods and Materials

Extraction of Genomic DNA from Tissue Samples

A Clontech Nucleospin Tissue Genomic DNA Extraction Kit was used to extract tissue samples as described below.
Preparation of Tissue Lysates

Tissue samples, about 25 mg, were cut into small pieces and placed in a 1.5 mL microcentrifuge tube. Aliquots of 180 µL of Genomic Digestion Buffer and 20 µL of Proteinase K were added to each tube. The samples were vortexed gradually to mix and incubated at 57°C overnight. The samples were then vortexed briefly for 30 seconds and placed back into the water bath for roughly 15 minutes before being centrifuged for 3 minutes at 1400 rpm. After centrifuging, the supernate was placed in a new sterile tube along with 20 µL RNase A. Each was vortexed for no more than 30 seconds and then allowed to sit for 2 minutes at room temperature. Aliquots of 200 µL of Genomic Lysis/Binding buffer and 200 µL of 100% ethanol were added to all tubes, which were then vortexed for 5 seconds.

Binding, Washing, and Eluting DNA from Tissue Lysates

Samples were transferred to a NucleoSpin spin column with collection tube and 640 µL of Lysate were added. After centrifuging for 1 minute at 10,000 rpm, the flow-through was discarded and 500 µL Wash Buffer 1 were added. Samples were centrifuged again at 10,000 rpm for 1 minute and flow-through discarded. An aliquot of 500 µL Wash Buffer 2 was added, the columns were centrifuged for 3 minutes at 14,000 rpm, with flow-through discarded. The collection tubes were discarded and the dry spin columns were placed in clean 1.5 mL tubes. A volume of 100 µL Elution Buffer was added to each sample, which were then incubated at room temperature for 1 minute. Samples were centrifuged for 1 minute at 14,000 rpm to collect DNA eluent. All samples were stored at -20°C until used.
Polymerase Chain Reaction Primers

Table 2 shows PCR primers used to confirm the presence of FeLV in the tissue samples.

Table 2: PCR Primers. Primers used in primary PCR.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>B5F</td>
<td>TCCCGAGTAACACCTCACCATTCCCA</td>
</tr>
<tr>
<td>B5R</td>
<td>AGCCTGGTGCCCTTAGGAACAGTCC</td>
</tr>
</tbody>
</table>

Polymerase Chain Reaction

Table 3 shows the general PCR mixture for one sample.

Table 3: Conditions used to Primary and Secondary PCR processes.

<table>
<thead>
<tr>
<th>Primary PCR</th>
<th>Secondary PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.5 µL H2O</td>
<td>19.5 µL H2O</td>
</tr>
<tr>
<td>2.5 µL 10x Advantage 2 PCR Buffer</td>
<td>2.5 µL 10x Advantage 2 PCR Buffer</td>
</tr>
<tr>
<td>0.5 µL dNTP</td>
<td>0.5 µL dNTP</td>
</tr>
<tr>
<td>0.5 µL Advantage 2 Polymerase Mix (50x)</td>
<td>0.5 µL Advantage 2 Polymerase Mix (50x)</td>
</tr>
<tr>
<td>0.5 µL Primer</td>
<td>0.5 µL Primer</td>
</tr>
<tr>
<td>1 µL tissue extract</td>
<td>1 µL tissue extract</td>
</tr>
<tr>
<td>24.5 µL total/ rxn</td>
<td>24.5 µL total/ rxn</td>
</tr>
</tbody>
</table>

Thermocycler conditions for both primary and secondary PCR are listed in Table 4 below. Once cycles were complete, samples were placed in -20°C refrigerator until used.

Table 4: PCR conditions used for thermocycler.

<table>
<thead>
<tr>
<th>Primary PCR</th>
<th>Secondary PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot start at 94°C for 1 minute</td>
<td></td>
</tr>
<tr>
<td>7 cycles: 94°C 25 sec 72°C 3 min</td>
<td>5 cycles: 94°C 25 sec 72°C 3 min</td>
</tr>
<tr>
<td>32 cycles: 94°C 25 sec 67°C 3 min</td>
<td>20 cycles: 94°C 25 sec 67°C 3 min</td>
</tr>
<tr>
<td>67°C for additional 7 minutes after final cycle</td>
<td></td>
</tr>
</tbody>
</table>
Purification of PCR Amplicons

A Clontech NucleoSpin Gel and PCR Clean-Up Kit was used to purify PCR products. Sample volumes were adjusted to 50 µL total volume, using 15 µL of PCR sample and 35 µL of water, then mixed with 100 µL of Buffer NT1. The 150 µL mixtures were loaded into cleanup columns with collection tubes and centrifuged for 30 seconds at 13,000 rpm; flow-through was discarded and columns were placed back into collection tubes. A volume of 700 µL of Buffer NT3 was added to the columns, which were then centrifuged for 30 seconds at 13,000 rpm. The flow-through was discarded, and this step was repeated. Samples were centrifuged for 1 minute at 13,000 rpm to remove any excess Buffer NT3. The spin tubes were placed in new clean 1.5 mL microcentrifuge tubes and incubated on a hot plate for 4 minutes at 70°C, with lids cracked. Aliquots of 20 µL of Buffer NE were then added and samples were incubated at room temperature for 1 minute. The columns were centrifuged for 1 minute at 13,000 rpm, allowing 1.5 mL tubes to collect PCR clean up eluent. Columns were discarded and the samples were placed in a -20°C refrigerator until used.

Digestion of DNA for Genome Walking Libraries

DNA templates were digested with restriction enzymes DraI, EcoRV, PvuII, and StuI according to the Genome Walker manual (Clontech). For each digestion, 25 µL of DNA extract were mixed with 8 µL of restriction enzyme (10 units/µL), 10 µL of buffer (10x), and 57 µL of deionized water. The tubes were inverted several times to mix, and incubated at 37°C for 2 hours. After vortexing gently for 10-15 s, the reactions were incubated overnight at 37°C.
Post-Digestion Purification

A NucleoSpin® Gel and PCR Clean-up Kit (Clontech) was used to purify the digested DNA samples. Spin columns were placed into collection tubes (2 mL) and 500-700 µL of sample digest were loaded. The columns were centrifuged for 30 s at 11,000 x g, and the flow-through was discarded. A volume of 700 µL Buffer NT3 was added to each and the columns were centrifuged for 30 s at 11,000 x g. The flow-through was discarded. The columns were centrifuged again for 1 min at 11,000 x g to remove Buffer NT3 completely, and then incubated for 5 min at 70°C. The columns were placed into new 1.5 mL microcentrifuge tubes, and 30 µL Buffer NE were added. After incubating at room temperature for 1 min, tubes were centrifuged for 1 min at 11,000 x g, and the eluent was collected. Samples were stored at -20°C until used.

Ligation of Adapter Sequences to Purified DNA Digests

Adapters were ligated to each sample of purified DNA digest according to the instructions in the Genome Walker kit (Clontech). The reaction mixtures contained 4.8 µL purified DNA digest, 1.9 µL Genome Walker Adaptor (25 µM), 0.8 µL of 10x Ligation Buffer, and 0.5 µL T4 DNA Ligase (6 units/µL). Samples were incubated at 16°C overnight in a thermal cycler, then incubated for 5 min at 70°C to stop the reaction. To each tube, 32 µL of TE buffer (10/1 pH 7.5) were added. The tubes were vortexed gently for 10-15 s, and then stored at -20°C until used.

Gel Electrophoresis

Gel electrophoresis was performed using 1.2% agarose gels. A volume of 4 µL of Gel Red intercalating nucleic acid stain was added to the dissolved gel mixture before pouring it into the gel tray. Gels were run at 90-110 V for about one hour. Time varied depending on the degree of separation of the bands. A Bio-Rad imaging system was used to read the gels.
Results and Discussion

To verify the presence of FeLV-B in the tissue samples collected, PCR using primers B5F and B5R was completed and gel electrophoresis was used. Figure 3 shows an image of the agarose gel obtained from this process. All lanes, except for the liver in lane 2, showed bands about 400 bp with regards to the reference ladders in lanes 4 and 8, confirming the presence of FeLV. The reason for the liver tissue sample not producing a band was unclear. Further PCR and gel pictures showed the virus within the tissue sample.

![Figure 3: Primary PCR from tissue samples. To verify the presence of FeLV in the extracted tissue samples, Primary PCR was done using primer B5F/B5R. Lanes are as followed: 1- Spleen, 2- Liver, 3- Mammary Tumor, 4- Reference ladders, 5- Lymph, 6- Kidney, 7- Brain.](image)

Post-PCR cleanup was done on all samples to remove any excess primers. Before sending the samples for sequencing at The University of Michigan Core DNA Sequencing Lab, the concentrations of the samples were adjusted to fit the sequencing criteria. By using Nucleotide Blast provided by National Center for Biotechnology Information website, the virus within the tissue samples was determined to match verified FeLV-B sequences more closely than FeLV-A, -C, or -T, as shown in Figure 4.
Figure 4: Sequencing Alignment. These images compare sequences obtained from extracted DNA from tumor sample. The sequences are aligned with known FeLV-A sequence, Rickard-A, and FeLV-B Glasgow sequence-B, K01209. The known sequences are imputed as Query and the sample DNA is the subject. These results show that our sample is more closely correlated with FeLV-B than it is to FeLV-A.

Sequencing chromatograms were visualized with the computer program Finch TV.

Figure 5 shows the sequence chromatogram obtained for the mammary tumor. Most of the peaks have good resolution with the exception of those within black boxes. These are peaks where the original sequence was given an “N” instead of a base pair due to multiple peaks being present. Because of this, it was thought that during PCR, multiple insertions were being amplified, which is consistent with the variable nature of the virus, and the fact that the majority of the sequencing peaks are clearly single-bases, with a good signal-to-noise ratio.
Figure 5: Chromatogram Sequence. Sequencing chromatogram for the amplicon of FeLV-B obtained from genomic DNA extracted from mammary tumor tissue sample.

Using the computer program ClustalX, both native and reverse complement sequences of tissue samples were aligned, Figure 6. Table 5 shows the tissue samples with corresponding reference numbers. The majority of the tissue samples line up very closely to each other, with a few exceptions. There are quite a few sites where there is no nucleotide base, but rather an “N.” These sites correspond to areas where multiple peaks were present in the chromatograms. The tumor’s samples contained more variation than the others and so further investigation into their sequencing was completed.
**Figure 6:** ClustalX alignment of tissue samples. Both native and reverse complement of the tissue samples were aligned together. See Table 5 for tissue sample with corresponding reference numbers.

**Table 5:** Sequence reference numbers with corresponding tissue.

<table>
<thead>
<tr>
<th>Reference Numbers</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>2432880/2432892</td>
<td>Spleen</td>
</tr>
<tr>
<td>243281/2432893</td>
<td>Spleen</td>
</tr>
<tr>
<td>2432882/2432894</td>
<td>Tumor</td>
</tr>
<tr>
<td>2432883/2432895</td>
<td>Tumor</td>
</tr>
<tr>
<td>2432884/2432896</td>
<td>Kidney</td>
</tr>
<tr>
<td>2432885/2432897</td>
<td>Kidney</td>
</tr>
<tr>
<td>2432886/2432898</td>
<td>Brain</td>
</tr>
<tr>
<td>2432887/2432899</td>
<td>Brain</td>
</tr>
<tr>
<td>2432890/2432902</td>
<td>Lymph</td>
</tr>
<tr>
<td>2432891/2432903</td>
<td>Lymph</td>
</tr>
</tbody>
</table>
For the tumor samples, the multiple peaks throughout the sequences were closely examined. For each pair, one of the nucleotides observed in the chromatogram was one that matched the other tissue samples. The overlapping nucleotide at each position differentiated the tumor samples from the other tissues. The sequences of the tumor samples were completed by replacing each “N” with one of the two nucleotide options. Those that would make the sequence different than the other samples were chosen. The original sequences of the tumor and the edited sequences are as follows:

2432894- Tumor Original
TTTCCCGAGTAACCTCACCTTCCTCCAAGGCAACGGAGGGCACCACCGTATATCTCTTTGTTATGTGCTCCATTGCCCTCTCTATGTACCCCTGTCACTCCGCAATGGGTAAAGTATGAGGACCGATATGGTTAATAATTTTGTGAGGGGACATATCTAGCTTTAAATGTCACTGACCCTCACAACTAAAACTAAGACGGAGGACCAAACCTTTTGTTTCTCCGCTGCTATCTGATCCGAACATTAAGCTGACTATATCCAGAAGTTGCCGAGGTTGNN

2432894- Tumor Edited
TTTCCCGAGTAACCTCACCTTCCTCCAAGGCAACGGAGGGCACCACCGTATATCTCTTTGTTATGTGCTCCATTGCCCTCTCTATGTACCCCTGTCACTCCGCAATGGGTAAAGTATGAGGACCGATATGGTTAATAATTTTGTGAGGGGACATATCTAGCTTTAAATGTCACTGACCCTCACAACTAAAACTAAGACGGAGGACCAAACCTTTTGTTTCTCCGCTGCTATCTGATCCGAACATTAAGCTGACTATATCCAGAAGTTGCCGAGGTTGNN

243289S- Tumor Original
TTTCCCGAGTAACCTCACCTTCCTCCAAGGCAACGGAGGGCACCACCGTATATCTCTTTGTTATGTGCTCCATTGCCCTCTCTATGTACCCCTGTCACTCCGCAATGGGTAAAGTATGAGGACCGATATGGTTAATAATTTTGTGAGGGGACATATCTAGCTTTAAATGTCACTGACCCTCACAACTAAAACTAAGACGGAGGACCAAACCTTTTGTTTCTCCGCTGCTATCTGATCCGAACATTAAGCTGACTATATCCAGAAGTTGCCGAGGTTGNN

2432895- Tumor Edited
TTTCCCGAGTAACCTCACCTTCCTCCAAGGCAACGGAGGGCACCACCGTATATCTCTTTGTTATGTGCTCCATTGCCCTCTCTATGTACCCCTGTCACTCCGCAATGGGTAAAGTATGAGGACCGATATGGTTAATAATTTTGTGAGGGGACATATCTAGCTTTAAATGTCACTGACCCTCACAACTAAAACTAAGACGGAGGACCAAACCTTTTGTTTCTCCGCTGCTATCTGATCCGAACATTAAGCTGACTATATCCAGAAGTTGCCGAGGTTGNN

Figure 7 shows the edited sequences aligned using ClustalX. Both native and reverse complement sequences of tissue samples were aligned with four reference sequences of FeLV-A, top four sequences, and three reference sequences of FeLV-B, 5th-7th sequences. When comparing tissue samples sequences to the references, it appears that they are more closely similar to the references of FeLV-B rather than FeLV-A; tissue samples do not have gaps of the
quences missing towards the beginning as do the FeLV-A references. Again, this is verification that the tissue samples contain feline leukemia virus that is more likely the B strain than the A strain. Positive verification can be obtained by viral interference assay.

**Figure 7:** ClustalX alignment of tissue samples. FeLV-A and FeLV-B reference sequences aligned with edited tumor sequences and other tissue sample sequences.

After review of the amino acid alignment, it appears the experimental sequences closely match those of FeLV-B and endogenous FeLV. The reading frame for the sequence change that contained the C to T change was read as CCT, which codes proline (P). This amino acid for the tumor sequences are boxed, in Figure 8. It appears that the change did not differentiate the sequence from the others. Had a C been placed in the sequence instead of a T, the reading frame would have produced CCC, which also codes for proline. This change was a silent mutation. It is marked as (a) in Figure 8.
Figure 8: Alignment of amino acids. Alignment of deduced amino acids from the tumor DNA sequences shown in Figure 7.

The two boxes labeled (b) on the amino acid sequence in Figure 8 also show a mutation, but they are not silent. When looking at the corresponding reading frame for these two sequences, they contained a change from A to G, coding for aspartic acid rather than asparagine. When looking at the natural isolate of FeLV-B obtained from the NCBI databases, the same change can be seen. To see how this amino acid change affects the virus, further research will need to be done.

Other changes in the nucleotide sequence did not produce any other noticeable changes in the amino acid sequences, leading to the conclusion that other silent mutations were made. Of note, the last box (labeled c) on the amino acid sequence in Figure 8 shows that of ten amino acid sequences aligned at that position, six different residues are present. When looking at the nucleotide sequences in Figure 7, the bases that code in this region are highlighted in a white box. The reason for the variance in the sequences is unclear, but the position of the mutation in the envelope gene is consistent with samples determined previously by other research groups.

Conclusion and Future Directions

FeLV-B sequences were successfully amplified from five of the six tissue extracts. New primers will be designed based on these sequencing results and will be used during the primer walking process in order to sequence the entire proviral insert. Sequencing the whole proviral

16
insert can lead to better understanding of how the insertion site is related to the progression of the symptoms.

To further investigate the primary PCR products, the samples will be cloned and transformed, followed by sequencing, as each isolated colony will contain a single variant of the virus. This will allow for testing of the hypothesis that multiple insertion sites were being amplified during this process.

Variations in the tumor samples were observed in the nucleotide sequences and when these variations were altered, mutations within the amino acid sequences were observed. Variations were not seen in the other tissue samples. This supports the original hypothesis that tumor samples would show mutations and/or rearrangements not observed in other tissues. Additional studies of the amino acid sequence will be done to better understand the possible mutations that occurred when altering the nucleotide sequence. Likewise, further work would be needed to determine the effect of these mutations on the disease progression and outcome.
References


