THESIS

DESIGN AND APPLICATION OF A DROPLET-DIGITAL PCR ASSAY FOR DETECTION OF THE STAT5B^{N642H} MUTATION IN FELINE T CELL NEOPLASIA

Submitted by

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ABSTRACT

DESIGN AND APPLICATION OF A DROPLET-DIGITAL PCR ASSAY FOR DETECTION OF THE STAT5B^{N642H} MUTATION IN FELINE T CELL NEOPLASIA

Lymphoma is a commonly diagnosed hematopoietic neoplasm in cats. Small Cell T-cell Epitheliotropic Intestinal Lymphoma (SCL) is the most reported subtype of lymphoma in cats. Cats with SCL are presented with non-specific clinical signs such as chronic vomiting, diarrhea, and weight loss. Diagnostic work-up often includes collection of intestinal biopsies with histopathology for diagnosis. SCL is characterized by infiltration of neoplastic lymphocytes into the intestinal epithelium and lamina propria of the small intestines. Neoplastic cells are small to intermediate in size and of T-cell origin. Diagnosing SCL can be challenging for pathologists because cats also commonly develop a condition called inflammatory bowel disease (IBD), which has an almost identical clinical presentation and similar histopathologic patterns. However, in IBD, the lymphocytic infiltration is often heterogeneous (termed "lymphoplasmacytic enteritis"). When histopathology results are inconclusive, assessment of expression with immunohistochemistry markers can help further characterize the cell population. Additionally, advancements have been made with lymphocyte clonality testing by PARR (PCR for Antigen Receptor Rearrangement), a DNA-based assay that evaluates T-cell receptor (TCR) and Immunoglobulin (Ig) gene rearrangements. Cats diagnosed with SCL demonstrate a clonal TCR result, while cats with IBD demonstrate a polyclonal TCR result. Unfortunately, there are still cases where histopathology and PARR results are equivocal.

Recent work in feline medicine has demonstrated that cats with SCL exhibit high expression of phosphorylated STAT5B with immunohistochemical staining on small intestinal

biopsy samples compared to cats with IBD. Importantly, one group detected a STAT5B^{N642H} mutation in cats diagnosed with SCL. In this study, 40% (17/42) of cats with intestinal lymphoma were classified as SCL by histopathology. A combination of Sanger sequencing and ARMS qPCR detected the STAT5B^{N642H} mutation in 29.4% (5/17) of cats with SCL. This work correlates to a comparable disease entity in people, monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL), which has reported the prevalence of the STAT5B^{N642H} mutation to be 22-57%.

Our group aimed to develop a droplet digital PCR (ddPCR) assay to detect wild-type and mutated STAT5B in cats. Our first aim was to design specific primers and locked-nucleic acid hydrolysis probes to detect and discriminate between wild-type and mutated STAT5B. The first step included analyzing data from control samples using wild-type DNA from cats without neoplasia and a positive control gene fragment block ("gBlock"). The second step included analyzing two cohorts of young cats (<6 years of age) without a diagnosis of lymphoid neoplasia to assess assay performance and determine if the mutated STAT5B could be considered a germ line polymorphism. The fractional abundance was calculated from the ddPCR data, estimating the percentage of mutated copies within a positive sample. The results of the first aim demonstrated that our ddPCR assay can distinguish between wild-type and mutated STAT5B with high sensitivity. Most young cats without a diagnosis of lymphoid neoplasia do not carry the STAT5B^{N642H} mutation. Two cats with marked lymphoplasmacytic enteritis had detectable mutated STAT5B^{N642H}.

The second aim was to evaluate the prevalence of the STAT5B^{N642H} mutation in cats with confirmed SCL. A sub-aim was to evaluate cats with CD4 T-cell leukemia to determine if this

mutation could be found in other forms of T cell lymphoid neoplasia. The results from this aim demonstrate that cats with SCL frequently carry the STAT5B mutation (66.7%). We also discovered that this mutation is not exclusive to cats with SCL, as almost half of the cats with CD4 T-cell leukemia also carry this mutation (47.7%).

These findings shed light on the prevalence of the STAT5B^{N642H} mutation in cats with SCL and CD4 T-cell leukemia. This data suggests potential implications for ddPCR mutation detection to help further differentiate and diagnose cats with T cell neoplasia versus those with inflammatory conditions (such as SCL versus IBD), and investigate novel therapies (i.e., JAK/STAT inhibitors). Further research is warranted to investigate other JAK/STAT pathway mutations, particularly in cats where the STAT5B^{N642H} mutation was not detected. Larger outcome studies should investigate the correlation of STAT5B^{N642H} mutation status and the fractional abundance to evaluate disease risk, treatment response, and survival.

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CHAPTER 1: INTRODUCTION

BACKGROUND

Study significance

Lymphoma is a commonly diagnosed hematopoietic neoplasm in cats.[1–4] Retrovirus-associated lymphomas were the most recognized before the control of the Feline Leukemia Virus (FeLV). [2,5] There are three subgroups of FeLV described (FeLV-A, FeLV-B, and FeLV-C) with the subgroup FeLV-B being overrepresented in cats with lymphoma.[6] FeLV-associated lymphomas are reported to be mostly of T-cell origin, although FeLV-associated B- origin lymphomas have also been described. [7] FeLV-associated lymphomas are associated with different anatomical forms such as multicentric (generalized lymphadenopathy +/- liver, spleen, and bone marrow involvement), mediastinal (mediastinal lymph nodes), gastrointestinal, and extranodal (renal, cardiac). [2,7,8] Non-FeLV-associated lymphomas now predominate among our cat populations, likely because of the development and incorporation of FeLV vaccination, FeLV testing, and knowledge of proper management strategies for FeLV-positive cats.[2,9,10] Currently, the most reported subtype of lymphoma diagnosed in cats is small cell T cell epitheliotropic lymphoma (SCL).[1,2,11,12] The pathogenesis of feline SCL is not clearly defined, although there are hypotheses that chronic antigenic stimulation within the gastrointestinal tract, such as from a food or infectious antigens, trigger an inflammatory infiltration of immune cells and cytokines. Eventually, this prolonged inflammation would lead to the deregulation of various cellular pathways (such as the JAK/STAT pathway) resulting in clonally expanded lymphocyte populations. [13,14] However, in-depth molecular and longitudinal outcome studies are warranted to further characterize this process. Cats with SCL are diagnosed at a median age of 13 years, and often present with non-specific clinical signs of chronic enteropathy which include anorexia, weight loss, vomiting, and diarrhea.[1,12,15]An extensive clinical workup is performed including

bloodwork, fecal exam testing, and abdominal imaging. Most patients often undergo a diet trial to rule out food-responsive enteropathy. Once all other common systemic diseases have been ruled out, a primary gastrointestinal disease is then further investigated. Histopathological lesions used to characterize SCL involve the infiltration of neoplastic lymphocytes, typically of small-to-intermediate size, within the intestinal epithelium and lamina propria of the intestinal villi. This infiltration may include the presence of intraepithelial nests and plaques, with the submucosa and muscularis often affected.[13,16] Endoscopic-guided biopsy samples of SCL cases can pose a challenge for pathologists to diagnose when the neoplastic lymphocytes do not extend beyond the mucosa. Therefore, this can lead to variations in the diagnosis based on the sample quality.[1,11,16–19] Alternatively, full-thickness intestinal biopsies can be collected in these patients, which may aid in a pathologist's diagnosis and interpretation. That said, endoscopic biopsies are often preferred in a clinical setting due to their less-invasive nature.[20] It has been reported that histopathology alone may not always provide a definitive diagnosis as the clinical presentation and biopsy findings can share features with inflammatory bowel disease (IBD).[11,16,17,19] IBD a non-neoplastic condition comprised of lymphoplasmacytic inflammation within the small intestines of cats.[21,22] As with SCL, the underlying pathogenesis of IBD is not clearly defined. It is hypothesized that IBD development is a result of interactions between environmental factors (such as diet and antigen exposure), the mucosal immune system, and enteric microbiome in susceptible cats.[21–23] Ultrasonographic findings of patients with SCL and IBD can also have overlapping features, with hypertrophy of the muscularis layer of the small intestine being reported as one of the most common findings. [24,25] Studies have shown that altered wall layering, jejunal lymphadenopathy, and abdominal effusion tended to be more prevalent in cats with SCL. [24–26] Immunohistochemistry (IHC) can be utilized in the assessment of lymphocyte phenotype

(CD3 for T cells, and CD20 for B cells) and distribution within the intestinal tract. [11,25] IHC can therefore aid in characterizing the severity and extent of lymphocyte infiltration, which can sometimes help the pathologist to differentiate between IBD and SCL.

When histopathology and IHC results are inconclusive, additional molecular testing can be pursued. PCR for Antigen Receptor Rearrangement (PARR) is a DNA-based assay evaluating the length of rearranged antigen receptor genes. In cases of SCL, PARR is particularly targeting the T-cell receptor (TCR) gamma loci's complementarity determining region (CDR3), which is unique to individual lymphocytes.[1,27,28] In heterogenous lymphocyte populations, the TCR gene products with be variable lengths, and would be consistent with a polyclonal TCR expansion. In contrast, neoplastic lymphocyte populations will have the same TCR gene length and result in a clonal TCR result by PARR. Clonal TCR results from PARR further support the diagnosis of lymphoma. [1,11,18,19,25,27–29] Previous studies indicate PARR's diagnostic sensitivity and specificity for the diagnosis of lymphoma/leukemia in cats to be close to 90%. [11,19,27,28] Nevertheless, cases may arise where histopathology and PARR results do not correlate, necessitating additional diagnostic tests to aid in equivocal cases.

Cats with SCL are described as having an indolent and slowly progressing disease course; however, most of the outcome and survival data are from studies in which cats underwent treatment after the initial diagnosis. [12,13,19] Clinical outcome studies comparing cats diagnosed with either SCL or IBD showed shorter overall median survival time for cats diagnosed with SCL compared to those with IBD. [16,19] The current treatment regimen for feline SCL has remained stagnant over the decades, with minimal development and investigation into new and alternative therapeutic strategies. Prednisolone, chlorambucil, or a combination of both therapies are the current mainstay treatments for cats with SCL.[12,15,20] The importance of being

able to diagnose SCL in cats, and for that matter neoplasia in general, may lead to a better understanding of etiopathogenesis, investigation of new targeted therapies for diagnosis and treatment, and improved patient care.

Signal Transducer and Activator of Transcription

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is highly conserved and plays a key role in cell signaling. [30–32] The signal transducer and activator of transcription (STAT) proteins are a group of transcription factors that mediate diverse biological processes, including cell immune responses, cell proliferation, and cellular differentiation.[30,31] Seven proteins are a part of the STAT family, with STAT5B being the most highly expressed in immune cells. [33–35] STAT5B is a latent cytoplasmic transcription factor that can be activated in response to a variety of cytokines, hormones, and growth factors that bind to their respective cell-surface receptor, and upon binding leads to phosphorylation of JAK proteins and subsequent phosphorylation and self-dimerization of STAT5B. [30,31,34,36] Once STAT5B is phosphorylated, it becomes dimerized and translocates to the cell nucleus leading to induction and upregulation of genes involved in cell proliferation and growth. STAT5B plays an essential role in T-cell development and activation. [30,31,36,37]

Mounting evidence indicates that dysregulation of the JAK/STAT pathway is implicated in various human cancers leading to uncontrolled cellular proliferation, anti-apoptotic responses, and angiogenesis.[30,32,34,35,38,39] Mutations in the JAK/STAT pathway have been reported in different forms of T-cell neoplasia in humans, such as monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL). [40–42] MEITL is a rarely diagnosed primary intestinal T-cell lymphoma, previously known as enteropathy-associated T-cell lymphoma type II. MEITL has overlapping

features with SCL in cats, in that MEITL is characterized by neoplastic small to medium in size lymphocytes that are T cell in origin which infiltrate the small intestines and demonstrate epitheliotropism.[43,44] In contrast to SCL in cats, MEITL in humans has an aggressive disease course with a high mortality rate. [40,45–47] In MEITL, mutations in the STAT5B transcription factor have been identified, with the N642H mutation being the most common, with a reported prevalence of 22-57%.[36,42,48] The outcome of patients with mutated STAT5B has been associated with poorer overall survival.[37,39–42,49] The STAT5B^{N642H} mutation is not exclusive to MEITL. It has been reported in other human T-cell neoplasms such as T-cell acute lymphoblastic leukemia, CD4+ and CD8+ T-cell large granular lymphocyte leukemias, T-cell prolymphocytic leukemia, and hepatosplenic T-cell lymphoma.[37,39,49] The STAT5B gene is found within the SH2 protein domain which is the phosphotyrosine-binding domain and is essential for STAT5B dimer formation. The STAT5B N642H mutation involves an A to C transversion (asparagine to histidine) single-nucleotide polymorphism (SNP) that results in constitutive phosphorylation leading to enhanced cellular proliferation and uncontrolled growth. [37,49–51] This mutation is considered a strong driver mutation.[37]

Recent work in feline medicine has demonstrated the STAT5B transcription factor to be highly expressed in feline SCL, compared to patients with IBD.[11] Importantly, one group has detected the same STAT5B^{N642H} mutation that was reported in human MEITL, in cats diagnosed with SCL. In this study, 40% (17/42) of cats with intestinal lymphoma were classified as SCL by histopathology. A combination of Sanger sequencing and ARMS qPCR was able to detect the STAT5B^{N642H} mutation in 29.4% (5/17) of cats with SCL.[52] Limitations to this study were that they did not evaluate tissue from cats without a confirmed lymphoma diagnosis (i.e. no negative

control samples), and only used histopathology as their diagnostic modality for diagnosing and categorizing lymphoma.

Droplet Digital Polymerase Chain Reaction

Droplet digital polymerase chain reaction (ddPCR) is a highly sensitive molecular technique enabling the detection of mutated sequences in clinical samples such as tumor DNA.[53] It has been suggested that ddPCR is a more reliable, sensitive, and robust technique compared to real-time or quantitative PCR (qPCR) techniques. [54–60] Unlike the relative measurements of DNA concentration obtained through qPCR methodologies, ddPCR enables the absolute quantification of target DNA molecules. The unique feature of ddPCR compared to quantitative PCR (QT-PCR), is the utilization of microfluidics to partition a DNA sample into approximately 20,000 oil-immersed nanoliter-sized droplets. This allows for increased assay sensitivity, especially regarding rare mutation detection.[53] An additional benefit of ddPCR is the capability to quantify the DNA concentration in a sample. In cancer mutation detection assays, this information can allow for assessment of the mutant fractional abundance (i.e. percentage of mutant allele copies, which can provide information on the percentage of mutant allele copies in a sample). Calculation of the fractional abundance is relevant to cancer research, especially for the detection of rare mutations. Cancerous tissue is often highly heterogeneous and cancer biomarkers vary across types of disease and stages of disease progression which complicates cancer detection and identification at early stages. Subclonal populations of cells within a tumor may contain a mutation that differs from the primary mutation, but the subclonal mutation could be correlated to a prognosis and/or a response to specific therapy regimens.

Our group aimed to develop a ddPCR assay to detect wild-type and mutated STAT5B in cats. We then aimed to apply this assay to evaluate the prevalence of the STAT5B^{N642H} mutation in cohorts of cats without T cell lymphoid neoplasia, and those with confirmed T cell lymphoid neoplasia.

PROJECT OVERVIEW

Given the prevalence and clinical importance of lymphoid neoplasia in the cat population, our project was driven by the need to deepen our understanding of the STAT5B^{N642H} mutation prevalence, a genetic alteration previously implicated in both human lymphoma/leukemias and cats with SCL. Recognizing the potential diagnostic and prognostic implications of this mutation, we aimed to further characterize its prevalence across different cohorts of cats. The first phase of our study focused on the development and validation of a sensitive and specific ddPCR assay designed to detect wild-type and mutant STAT5B in cat DNA samples. This involved the optimization of assay conditions and assessment of its analytical performance using both positive and negative control samples. The negative control DNA was collected from blood and tissue samples from young cats (<6 years of age) without a confirmed lymphoma/leukemia diagnosis. The positive control for each assay was a custom-synthesized gene fragment (gBlock) designed to have the mutated gene sequence. These controls were selected to evaluate assay performance and to provide information about whether the STAT5B^{N642H} SNP could be considered a germ line polymorphism in cats. We hypothesized our ddPCR would be able to distinguish between wild-type and mutant STAT5B, and we would not detect the STAT5B^{N642H} mutation in young cats without lymphoma/leukemia.

The second phase of our project consisted of analyzing tissue and peripheral blood samples obtained from cats with confirmed diagnoses of SCL and CD4 T cell leukemia,

respectively. We aimed to investigate the prevalence and distribution of the STAT5B^{N642H} mutation within these specific disease cohorts. We hypothesized a high frequency of detection for the mutant allele in intestinal biopsy samples obtained from cats diagnosed with SCL, aligning with previous reports. Furthermore, we anticipated that the STAT5B^{N642H} mutation is not exclusive to SCL, and cats diagnosed with CD4 T cell leukemia would also carry this mutation.

Our overarching goal was to provide information regarding ddPCR assay design for mutation detection, in addition to insights into STAT5B^{N642H} mutation prevalence and distribution in cats with SCL and CD4 T cell leukemia, which may lead to improved diagnostic strategies and therapeutic interventions.

SPECIFIC AIMS

Aim 1: Design a droplet-digital PCR (ddPCR) assay for detecting STAT5B in cats and distinguishing between wild-type and mutant STAT5B.

Hypotheses:

1. Our ddPCR assay will successfully differentiate between mutant and wild-type STAT5B.
2. Young cats under 6 years old exhibiting peripheral lymphocytosis and demonstrating polyclonal TCR results via PARR will not carry mutated STAT5B.
3. Young cats under 6 years old, regardless of an enteritis diagnosis, and exhibiting polyclonal TCR results via PARR will similarly not carry mutated STAT5B.

Objectives:

1. Develop specific ddPCR primers and locked-nucleic acid hydrolysis probes to target the STAT5B allele, along with a gene fragment block ("gBlock") serving as a mutant positive control.
2. Analyze samples from young cats (<6 years of age) to determine the prevalence of mutations in these cohorts and assess the ddPCR assay performance.

Aim 2: Analyze and characterize the prevalence of the STAT5B mutation in cohorts of cats diagnosed with small cell T cell epitheliotropic lymphoma (SCL) or CD4 T cell leukemia.

Hypotheses:

1. Our ddPCR assay will detect the STAT5B mutation in cats diagnosed with SCL at a high frequency.
2. The STAT5B mutation will not be exclusive to cats diagnosed with SCL and will also be frequently detected in cats diagnosed with CD4 T cell leukemia.

Objectives:

1. Analyze samples from cats diagnosed with SCL via histopathological examination by two pathologists and demonstrate clonal TCR results via PARR for the STAT5B mutation using ddPCR.
2. Analyze samples from cats diagnosed with CD4 T cell leukemia via flow cytometry and demonstrate clonal TCR results via PARR for the STAT5B mutation using ddPCR.
3. Assess the allele frequency calculated from the ddPCR data and correlate it with patient data.

CHAPTER 2: DROPLET DIGITAL PCR ASSAY DEVELOPMENT

MATERIALS AND METHODS

DNA samples

DNA from tissue biopsy samples was collected retrospectively from formalin-fixed paraffin-embedded (FFPE) blocks at Colorado State University's (CSU) Veterinary Diagnostic Laboratory (VDL). DNA from peripheral blood samples was also collected retrospectively from CSU's Clinical Hematopathology laboratory. Genomic DNA (gDNA) was extracted using a QIAamp DNA Mini Kit (QIAGEN according to the manufacturer's instructions) and DNA quantification was performed using a Nanodrop One (Thermo Fisher: ND-ONE-W).

To achieve a theoretical 1 in 10,000 sensitivity for the droplet digital PCR assay, ≥ three times the number of the desired wild-type alleles were screened to ensure 95% confidence that at least one mutant allele would be detected ("Rule of Three" BioRad Rare Mutation Detection/Best Practices Guidelines (Bulletin 6628 Rev A)). Therefore, at least 30,000 copies of wild-type alleles would need to be screened. The cat genome size, estimated at 2.7 billion base pairs (Bbp), is approximately 88% the size of the human genome.[61] Therefore, a total of 160ng cat genomic DNA (gDNA), which equals about 54,000 STAT5B genome copies/equivalents, was screened in duplicate reactions for accuracy. A concentration of 160ng per sample was chosen to supply an ample buffer of wild-type gene copies screened.

PCR Primers, Probes, and gBlock™

The single-nucleotide polymorphism assay was designed according to information provided in the Bio-Rad "Rare Mutation Detection/Best Practices Guidelines" (Bulletin 6628 Rev A). The test involved a single set of primers and two competitive hydrolysis-locked nucleic

acid (LNA) probes (synthesized by Integrated DNA Technologies) (Table 1). The hydrolysis probes (Figure 1) were designed for allele-specific ddPCR: FAM-labeled, complementary to the mutant allele, and HEX-labeled, complementary to the wild-type allele.

A gene fragment block (gBlock™), containing the mutant amplicon, was used as positive control for the 6-FAM probe (Table 1). The gBlock™ is a sequence-verified, double-stranded DNA fragment (custom synthesized by Integrated DNA Technologies, IDT). The gBlock stock solution was diluted (5e-8 dilution _~2,000 copies/ul) for appropriate positive control droplet concentrations.

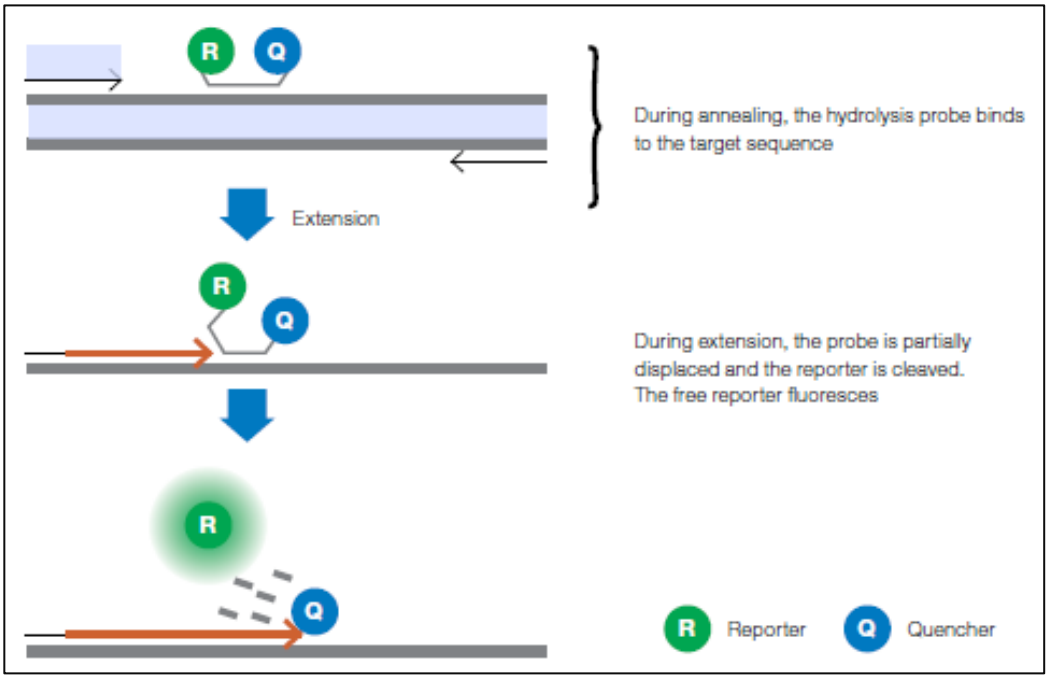


Figure 1. Hydrolysis of TaqMan probes during qPCR amplification process. When a target sequence bound by a TaqMan probe is amplified during PCR, the reporter dye will emit a fluorescent color which can then be detected and measured. An increase in amplified template yields an increase in fluorescence (I. Bio-Rad Laboratories. (Life Science Group), pp. 1-100.).

Table 1. Sequences of ddPCR primers, probes, and gBlock.

Primers	Sequence	Modifications
Pq3un (forward)	TGATTATTCTGTTTGTCGCTCT	na
6 (reverse)	ATGGAGAAGTCTCTGGTGGT	na
Probes		
fst5bwt_17	CATC+A GA+T +T+CC A+AA ACA*	HEX/Iowa Black FQ
fst5bmt_17	CATC+A GA+T +G+CC A+AA ACA	6-Fam/Iowa Black FQ
Gene fragment block "gBlock"		
220104_RB_gBlock_STAT5B	GTTTAGAATATGATTATTCTGTTTGTCGC TCTAGAGGAAAGGATGTTTTGGCATCTG ATGCCTTTTACCACCAGAGACTTCTCCA TCCGGTCCCTGGCTGACCGCTTAGGGG ACCTGAATTACCTTATCTACGTGTTTCC#	na

*Nucleotides preceded by a "+" mark are locked. #140bp STAT5B gBlock sequence containing the 76bp N642H ddPCR amplicon. Primer positions/sites are underlined.

PCR for antigen receptor rearrangements (PARR)

PARR was performed on all DNA samples extracted from both peripheral blood and formalin-fixed paraffin-embedded tissue samples to assess the clonality of T-cell receptor gamma (TRG) gene rearrangements. PARR was performed as previously described.[27] Inclusion criteria were cases that had a confirmed polyclonal TRG result by PARR.

Droplet Digital Polymerase Chain Reaction

Droplet Digital Polymerase Chain Reaction (ddPCR) was performed with a PCR reaction volume of 20ul, using the ddPCR™ Supermix for Probes (no dUTP) (Bio-Rad, Hercules, CA). Reaction conditions included 11µl of ddPCR Probe Supermix, forward and reverse primers at 900nM each, probes at 250nM, and template DNA. Each plate contained two non-template control wells (NTC) for quality control and four wild-type only wells. These wells were used to

determine the false positive rate (FPR) for each experiment. As the false-positive rate (FPR) is a function of the amount of sample loaded, wild-type-only wells were run at concentrations like (equivalent to/appropriate for) the test samples. An aliquot of diluted gBlock was spiked into the wild type gDNA control and run in duplicate on each ddPCR plate. In addition to acting as a mutant allele positive control, these wells were used to identify expected cluster positions and to inform manual thresholding.

Droplets were generated with the AutoDG, Automated Droplet generator (BioRad). Amplifications were performed on a MiniAmp Plus thermocycler (ABI/ThermoFisher) using the following conditions: 1 cycle of 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 59 °C for 1 min, and 1 cycle of 98 °C for 10 min. Samples were scored on a QX200 Droplet Reader (BioRad), and analysis was performed with Quantasoft software (Ver. 1.7.4.0917) (Figure 2).

Quantasoft software measures the number of droplets positive for each probe (FAM_mutant STAT5B; HEX_wt STAT5B) in each sample and calculates the fraction of positive droplets by a Poisson algorithm to determine the concentration of the target. The software uses concentration data to determine the fractional abundance (FA) of mutant to wild type template. The FA is calculated as the percentage ratio between the number of mutant DNA molecules (a) and the number of mutant (a) plus wild type (b) molecules (fractional abundance: (a/a+b)).

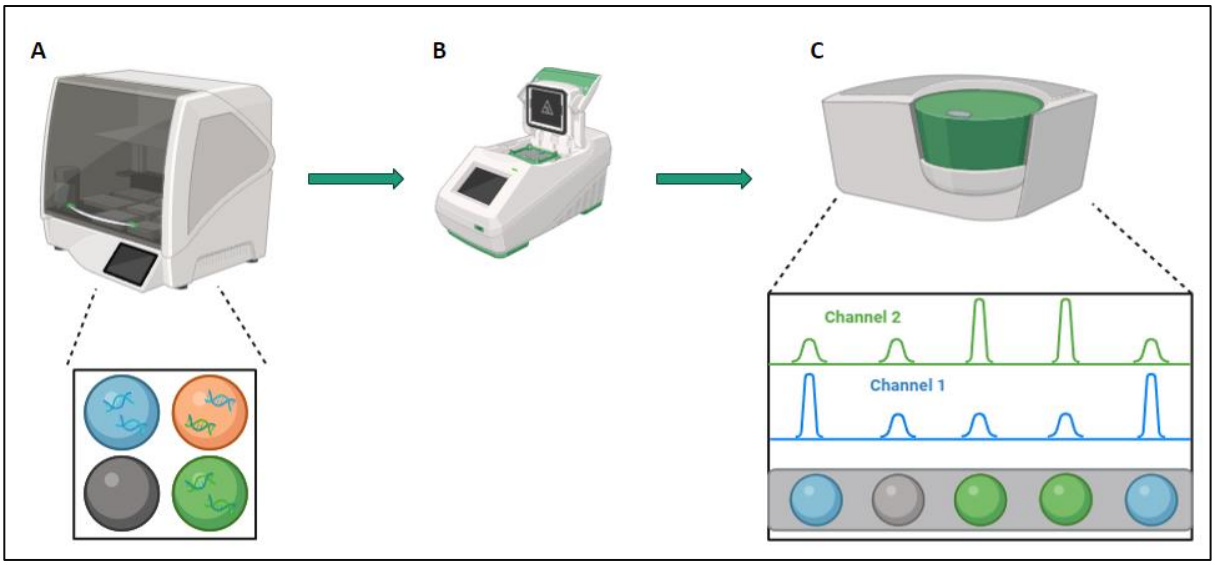


Figure 2. Process in analyzing samples with ddPCR. **A.** Samples are put in a 96-well plate and added to the AutoDG, Automated Droplet generator (BioRad). Each sample, containing target and background DNA, is partitioned at random into ~20,000 nanoliters sized droplets using oil immersion. Blue droplets = FAM+ (mutant DNA), Green droplets = HEX+ (wild-type DNA), Orange droplets = FAM+/HEX+, Grey droplets = Background DNA **B.** The sample plate is transferred to a thermocycler for PCR. **C.** After PCR, fluorescent readings are measured by the QX200 Droplet Reader (BioRad) for each droplet in two channels based on color. "Positive" droplets will contain at least one copy of the target DNA molecule and will exhibit a higher intensity of fluorescence. "Negative" droplets will contain zero copies of the target DNA molecule and will exhibit no fluorescence.

Limit of detection

The limit of detection (LoD) is defined as the lowest mutant concentration that can be reliably distinguished from the wild-type only control. For determination of the assay's LoD, serial dilutions of positive control gBlock (1:4 to 1:4096) in a constant background of wild type gDNA was performed. Two sample plates were analyzed, one containing a "fresh" (peripheral blood) control DNA sample and one containing a FFPE (intestinal biopsy) control sample, since these were the two sample types used for our study design. PCR reactions were prepared according to the ddPCR reaction conditions previously detailed. The FA and droplet concentrations were calculated and plotted. The false positive threshold of the assay was determined as the upper limit of the mutant concentration error bars in the wild-type-only wells.

LoD was calculated as the lowest mutant concentration where the lower limit of the error bar does not cross the false positive threshold. The first concentration value above the threshold represents the lowest detectable concentration.

STAT5B mutation status

The criteria for determining the presence of the STAT5B mutant allele (FAM+ droplets) in a sample included manual gating of the spiked mutant and wild-type control wells to determine manual cluster positions. Next, gating of only the wild-type control wells was performed to determine the false positive threshold of the plate (using the Poisson 95% confidence interval determined by the software). A STAT5B mutation status was reported as "present" if the lower limit of the error bar of the sample was greater than three times the upper error bar of the wild-type control threshold. The positive (FAM+) droplets on the 2-D plot must also show fluorescence (i.e. clustering) consistent with the positive control in each plate. If a sample did not meet these criteria, it was reported as "absent" for the STATB mutation.

Patients

Two cat cohorts were included in ddPCR development to assess the performance of the assay. These two cohorts were selected as they had an adequate heterogenous lymphoid population to evaluate, and based on clinical and additional diagnostic data, had no previous diagnosis of T cell neoplasia. The reported median age of cats diagnosed with lymphoma is 13 years old. Therefore, we elected to evaluate cats less than 6 years old, as they were less likely to have T cell neoplasia. Inclusion criteria for the first cohort of cats included those that were <6 years of age with a peripheral lymphocytosis (>6,000/ul) on bloodwork, heterogenous lymphoid population by flow cytometry, and polyclonal TCR results by PARR. Peripheral blood was

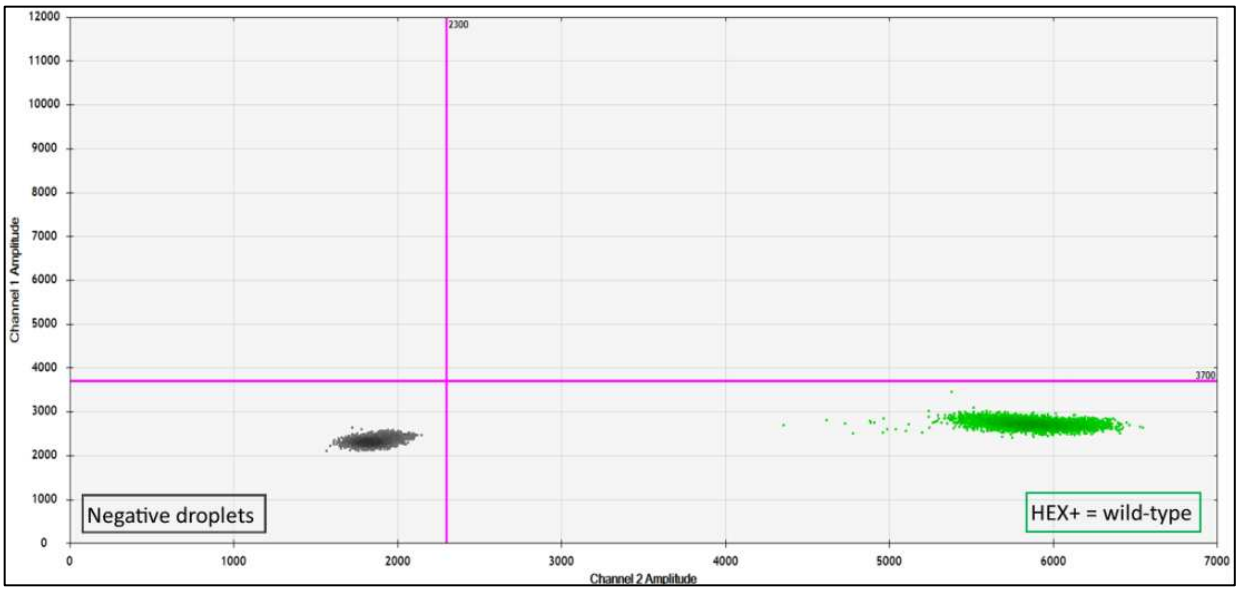
analyzed in this group. Inclusion criteria for the second cohort of cats included cats that were also <6 years of age with intestinal biopsy samples that were diagnosed with no significant findings or enteritis by a pathologist and had polyclonal TCR results by PARR.

RESULTS

Validation of ddPCR for STAT5B^{N642H}

The ddPCR assay for STAT5B can generate four expected clusters with a template of target PCR amplicon and wild type gDNA. The four visible clusters are double negative partitions that contain no amplified targets (background gDNA), single positive partitions for mutant (blue) or wild-type (green) and double positive partitions that contain a positive signal for both mutant and wild type targets. Double positive clusters tend to form an arc conformation that spans the two single positive clusters, which may be suggestive of variable amounts of targets (mutant or wild type) in each droplet. Manual thresholding was performed to separate the four clusters, and the quantification of each target was calculated by Quantasoft software. Examples of a STAT5B mutation assay that is both “absent” and “present” for the STAT5B mutation are shown in Figure 3.

A.



B.

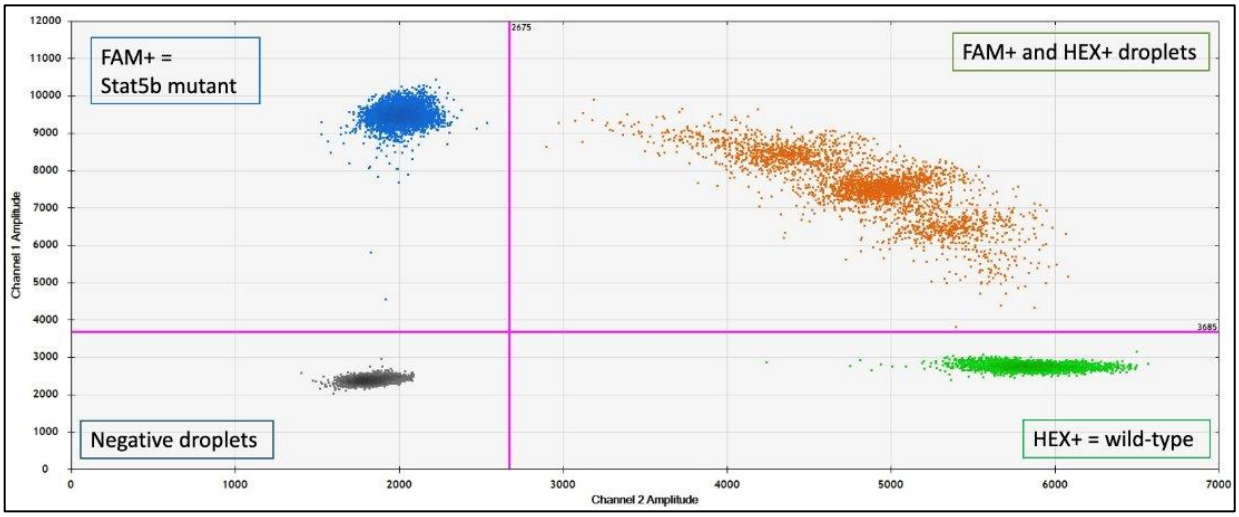


Figure 3: 2-D fluorescence amplitude plots for the STAT5B mutant detection. **A**. Example of a sample called "absent" for the STAT5B mutant. **B.** Example of a sample called "present" for the STATB mutant. The grey droplets represent background (non-amplified) DNA. The green cluster represents droplets that are positive for wild-type only DNA (HEX probe). The blue cluster represents droplets that are positive for mutant-only DNA (FAM probe). The orange cluster represents droplets that are positive for both wild-type and mutant DNA.

Limit of detection

All samples were evaluated using the Quantasoft software. Figure 4 displays the results from the serial dilutions performed and the LoD for the two STAT5B ddPCR assays. The data showed precision and linearity down to dilutions of 1:4096. The LoD for the "FFPE" plate

(Figure 4A) established an FA>0.05%, that is, it can detect 5 positive events of a total of 10,000 events. The LoD for the "Fresh" plate (Figure 4B) established an FA >0.02%, that is, it can detect 2 positive events of a total of 10,000 events.

Simple linear regression was performed to assess Pearson's r correlation between the STAT5B measured mutant concentration value obtained by ddPCR and the calculated (i.e. expected) mutant concentration values for both the FFPE (Figure 5A) and Fresh (Figure 5B) plates. Analysis of the results (GraphPad Prism 9.5.1) showed that ddPCR assays yielded comparable mutant frequency values which agree well with the expected values (Pearson correlation coefficient, $R^2 = 0.99$).

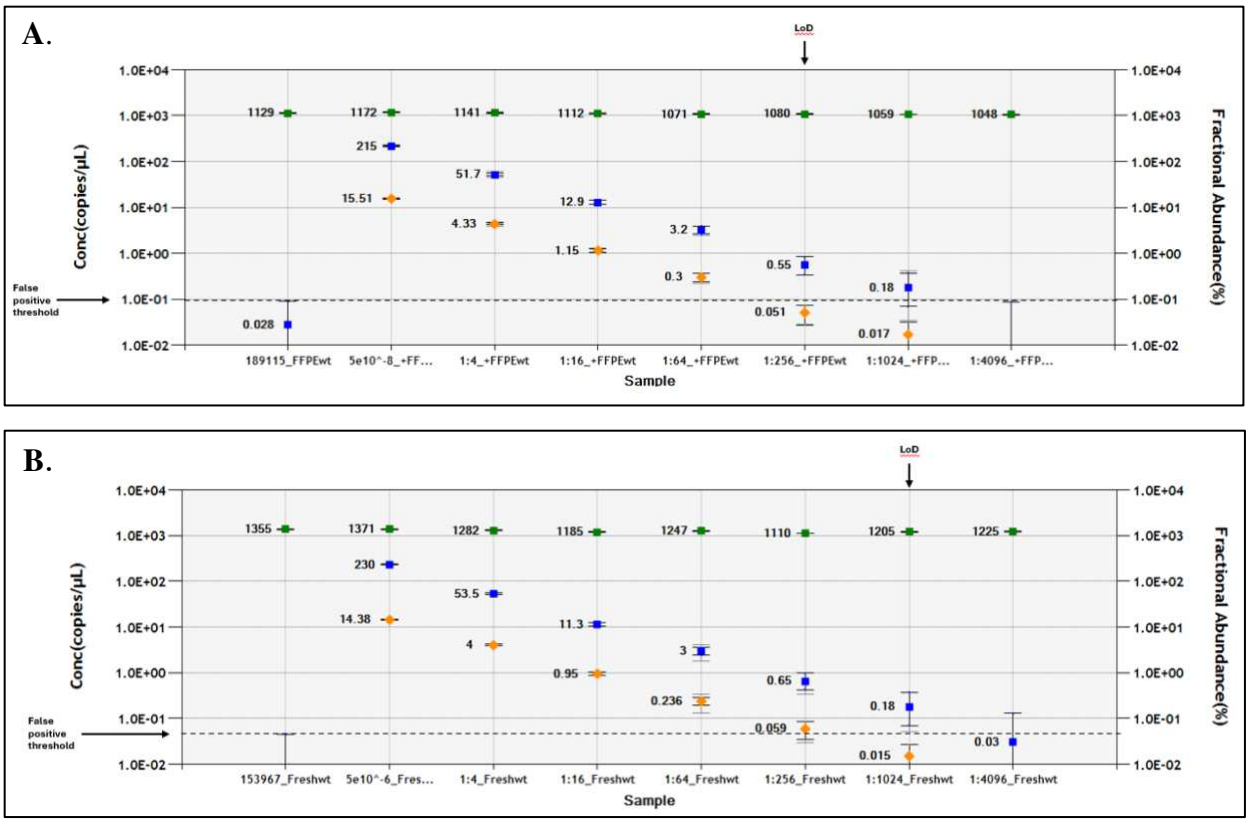


Figure 4. Limit of detection for ddPCR. **A.** FFPE gDNA control. **B.** Fresh gDNA control. Wild-type (wt) template concentration in copies per ul (green), fractional abundance of mutant to wt template (orange), and mutant template concentration (blue) are reported for each dilution of STAT5B on the genomic wt background. The false positive threshold (dashed line) was determined as the upper limit of the mutant concentration error bars for the "wt only" control. The value of the LoD was equal to the lowest dilution value where the lower error bar of the concentration of the mutated target remained above the threshold.

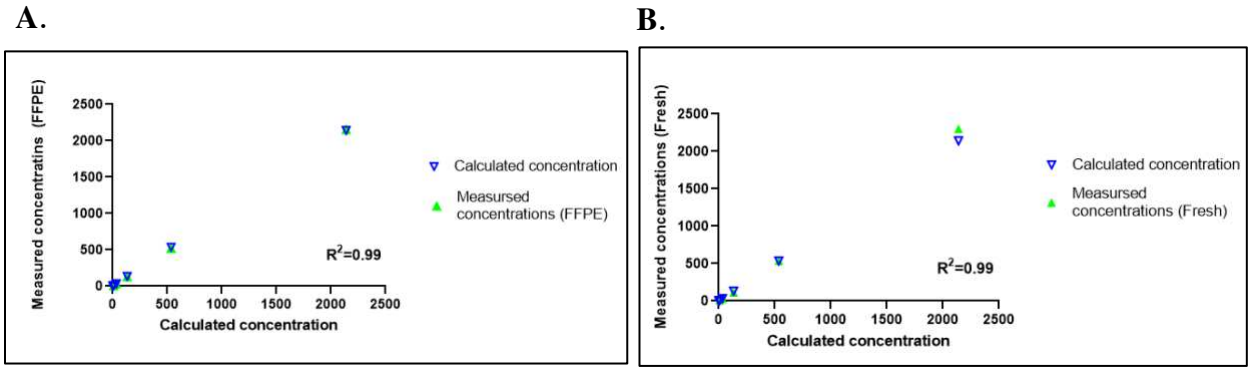


Figure 5: Simple linear regression graphs for the measured and calculated mutant STAT5B concentrations. **A**. Simple linear regression comparison graph for the FFPE LoD assay. **B**. Simple linear regression comparison graph for the Fresh LoD assay.

Criteria for positive and negative STAT5B status by ddPCR

The first step was to evaluate the no-template control (NTC) wells in each plate to assess quality control. This was followed by evaluating the wild-type and spiked gBlock wells to set manual thresholding for droplet determination. The wild-type only wells were assessed for quality control and to set the false positive threshold. In order to increase assay specificity and confidence for calling a sample positive, the lower 95% confidence interval of mutant concentration in a sample must be greater than three times the false positive threshold. Each sample was evaluated individually. Figure 6 provides an example of a sample determined positive for the STAT5B mutation by the assay. The lower error bar of the 95% confidence interval of the mutant concentration was greater than three times the upper error bar of the 95% confidence interval (false positive threshold) of the mutant concentration in the wild-type only wells (Figure 6A). On the 2-D fluorescence graph, this sample also demonstrates appropriate clustering and fluorescence for the mutant positive (FAM+/blue) droplets (Figure 6B).

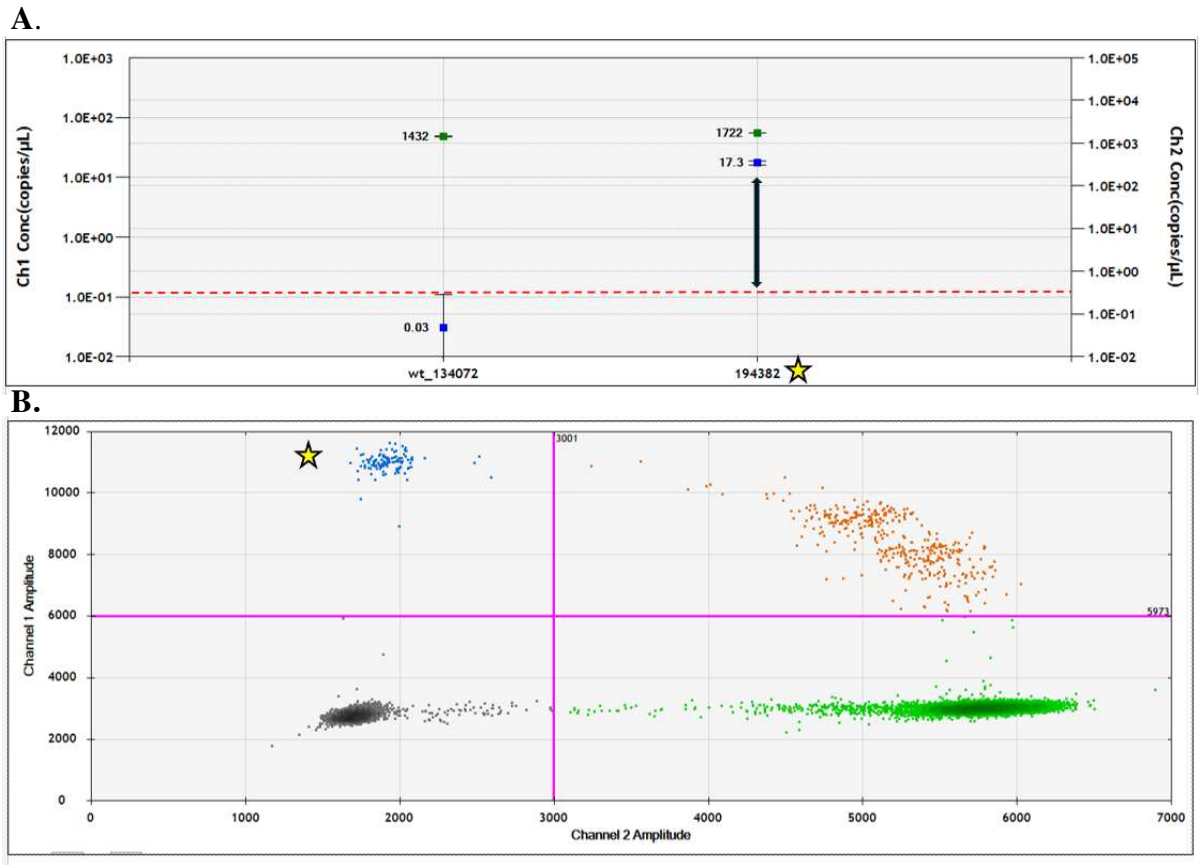


Figure 6. Criteria for determining a positive STAT5B sample by ddPCR. **A.** Demonstrating the lower reference interval of the sample (yellow star) mutant concentration is greater than three times the upper reference of the wild-type control mutant concentration (black arrow). **B**. Demonstrating the FAM positive (mutant) droplets demonstrate appropriate clustering and fluorescence for the sample (yellow star).

Evaluation of the ddPCR assay using cat gDNA samples

A total of 35 patients met the inclusion criteria for evaluating the peripheral blood in cat young cats with lymphocytosis. The median age at presentation was 2.58 years (range, 0.5-6). Of these, all (0/35) were negative for the STAT5B mutation by ddPCR. 28 patients met the inclusion criteria for evaluating intestinal biopsy samples in young cats with no suspicion of neoplasia. The median age at presentation was 2.1 years (range, 2.1-5.2). Of these 7% (2/28) were positive for the STAT5B mutation by ddPCR, with the remaining 93% (26/28) being negative. One of the positive STAT5B mutation cats was a 5-year-old MC DSH diagnosed with mild lymphoplasmacytic and suppurative enteritis and was polyclonal by PARR. The STAT5B

mutational FA was 0.13% by ddPCR. The second cat that was positive for the STAT5B mutation was a 2.6-year-old MC Siamese diagnosed with severe lymphoplasmacytic duodenitis and was polyclonal by PARR. The STAT5B mutational FA was 0.09% by ddPCR. See Table 2 for a comprehensive patient data chart.

Table 2. Clinical features of cats.

Characteristics	Peripheral Blood	Intestinal Biopsies
Number of cats (n)	35	28
Age (median, years)	2.58 (0.5- 6)	2.1 (0.8-5.2)
Sex		
MC	13	17
FS	20	7
FI	2	2
MI	-	2
Breed		
Mix breed (DSH, DMH, DLH)	31	21
Purebred (ASH, MCC, SPHX, PERS, SIAM, SAV, RUSSB, RAG, CAL, NORFOR)	4	7
Average total lymphocyte count	12,200 (6,600-32,240)	-
Histologic diagnosis		
*Enteritis	-	23
Fibrosis	-	1
No significant lesions	-	4
PARR (TCR)		
Polyclonal	35	28
Clonal	0	0
STAT5B mutation status		
Present	0	7% (2/28)

Absent	0% (0/35)	93% (26/28)

Abbreviations: PARR, PCR for antigen receptor rearrangements; FI, female intact; FS, female spayed; MI, male intact; MC, male castrated. Breed abbreviations: ASH, American Shorthair; CAL, Calico; DLH, Domestic Longhair; DSH, Domestic Shorthair; DMH, Domestic Medium Hair; MCC, Maine Coon Cat; NORFOR; Norwegian Forest; PERS, Persian; RAG, Ragdoll; RUSSB, Russian Blue; SAV, Savannah; SIAM, Siamese; SPHX, Sphynx

*Subcategories of enteritis include neutrophilic, eosinophilic, lymphoplasmacytic, and mixed.

DISCUSSION

Droplet digital PCR (ddPCR) has emerged as a sensitive diagnostic tool, particularly valuable in the detection of rarely mutated alleles, commonly seen in cancer mutation analysis. The primary objective of this study was to develop a ddPCR assay capable of detecting and distinguishing between wild-type STATB and mutant $STATB^{N642H}$ in cats. Custom-synthesized primers and locked nucleic acid (LNA) hydrolysis probes were employed to specifically bind to the STAT5B gene sequence in cat DNA. LNA hydrolysis probes offer several advantages in ddPCR, including enhanced specificity and sensitivity due to their modified nucleotides, reducing non-specific binding and false positives, and enabling efficient target detection even at lower concentrations. Moreover, LNA probes improve the discrimination of single nucleotide polymorphisms (SNPs) owing to their high specificity.

The results of the first part of the study demonstrated the reliable detection and differentiation of mutant and wild-type STAT5B alleles using ddPCR. The appropriate clustering and separation of fluorescent droplets in the assay for both mutant (FAM+) and wild-type (HEX+) droplets were observed. The greater separation of clustering in droplets containing both mutant and wild-type alleles (FAM+/HEX+) was hypothesized to be a result of variation in allele numbers (i.e. concentration) in each droplet affecting fluorescence readings.

In the subsequent phase, the assay's sensitivity was evaluated through the determination of the limit of detection (LoD). Two LoD assays were performed for each sample type—formalin-fixed paraffin-embedded (FFPE) tissue and peripheral blood ("fresh" tissue)—yielding fractional abundances (FA%) of 0.05% and 0.02%, respectively. It's important to acknowledge that formalin fixation can induce DNA fragmentation and various forms of DNA damage, such as DNA-DNA and DNA-protein crosslinks, along with cytosine deamination. Moreover, strong PCR inhibitors, including detergents found in lysis buffers, may disrupt droplet cluster locations, leading to decreased sensitivity. Additionally, the presence of excess degraded DNA can exacerbate PCR inhibition, further diminishing sensitivity. Therefore, the difference in LoD values in the FFPE and fresh tissue assays could be a result of formalin fixation effects, as the wild-type DNA is used to set the false positive threshold. In Figure 4 we can see that the false positive threshold is higher in the FFPE plate than the fresh tissue plate, which ultimately affects the LoD determination. A recommendation from the BioRad ddPCR manual suggests employing uracil DNA glycosylase (UDG) treatment on FFPE samples before analysis to mitigate false-positive events resulting from formalin fixation-induced cytosine deamination. However, it is noteworthy that UDG treatment is effective only for specific SNP mutations and does not apply to the STAT5B^{N642H} mutation (A>C transversion) targeted in our assay. Furthermore, UDG treatment of samples for the STAT5B ddPCR could potentially convert true mutants into amplicons that neither the wild type nor mutant probe would bind to, theoretically reducing the frequency of mutant copies in genuine positive samples.

Further evaluation of the assay's performance in a cohort of "negative" control cats aimed to assess its effectiveness in clinical samples. The ddPCR assay did not detect mutant STAT5B^{N642H} in any peripheral blood samples analyzed from young cats with peripheral

lymphocytosis. However, the assay did detect mutant STAT5B^{N642H} in 7% (2/28) of samples from young cats with enteritis diagnosed by histopathology, while the remaining 92% (26/28) were negative. Another goal of evaluating a cohort of young cats without evidence of neoplasia was to demonstrate that the STAT5B N642H mutation is not a germ line polymorphism. The ideal sample cohort for "true" negative control cats would be those without clinical signs of chronic enteropathy or any histopathologic abnormalities detected on intestinal biopsy samples. However, obtaining such a cohort, even among young cats, is challenging from a clinical and diagnostic standpoint. This challenge is illustrated in our study, where only 14% (4/28) of cats in this cohort had no significant lesions on their intestinal biopsy samples by histopathology, while 82% (24/28) exhibited some degree of enteritis.

The two cats in the intestinal biopsy group that were positive for mutant STAT5B^{N642H} were followed up clinically. One of these cats, a 2.6-year-old male Siamese, initially presented to their primary veterinarian with a history of vomiting and gagging. Endoscopic biopsies revealed "severe lymphoplasmacytic duodenitis" upon histopathological examination. Polymerase chain reaction (PCR) for antigen receptor rearrangements (PARR) performed on DNA from the FFPE biopsy tissue showed polyclonal results TCR results. Subsequently, ddPCR conducted on DNA from the same FFPE biopsy tissue yielded a fractional abundance of 0.13%. The cat received a diagnosis of inflammatory bowel disease and underwent treatment with metoclopramide and prednisolone. Follow-up on the patient two months post-diagnosis and treatment indicated significant clinical improvement, although no further diagnostics were performed, and the patient has since been lost to follow-up.

The second cat, a 5-year-old male domestic shorthair, presented to their primary veterinarian for chronic vomiting, diarrhea, and weight loss. Endoscopic biopsies revealed "mild

lymphoplasmacytic and suppurative enteritis" by histopathological examination. PARR performed on the FFPE biopsy tissue demonstrated polyclonal TCR results. ddPCR conducted on DNA extracted from the same FFPE biopsy tissue yielded a fractional abundance of 0.09%. The cat received a diagnosis of inflammatory bowel disease and underwent treatment with prednisolone, omeprazole, omega-3 fatty acids, and a strict diet of Royal Canin rabbit formula. Follow-up conducted by the submitting veterinary clinic reported consistent management with a local Internal Medicine specialist over the last 6 years, during which the cat exhibited no episodes of vomiting or diarrhea and attained an appropriate body condition score. No further diagnostics were performed at the time of follow-up.

These cases present a challenging diagnostic scenario. The high sensitivity of the ddPCR assay suggests the potential for detecting mutations before clonal TCR rearrangements can be detected by PARR or small cell lymphoma (SCL) diagnosed by histopathology. Therefore, it is conceivable that these patients may eventually develop SCL, however this was not demonstrated in either case. Given their favorable clinical response to treatment initiation, neither underwent repeated diagnostic testing (such as intestinal biopsy, PARR, STAT5B) to assess disease progression. It raises questions about the impact of early treatment initiation on disease maintenance and progression. Additionally, while the $STAT5B^{N642H}$ mutation is considered a strong driver mutation, the multifactorial nature of cancer development suggests the involvement of other epigenetic and genetic factors, such as additional mutations in the JAK/STAT pathway. Furthermore, utilizing fractional abundance as a cutoff point for determining clinical significance with the STAT5B ddPCR assay warrants further investigation, requiring correlation with additional clinical and diagnostic information.

In conclusion, the STAT5B ddPCR assay demonstrated utility in detecting the STAT5B allele and distinguishing between wild-type and mutant STAT5B alleles in cats. The assay exhibited high sensitivity for mutant detection in both fresh and FFPE tissue samples. However, the diagnostic and prognostic significance of the STAT5B^{N642H} mutation requires further elucidation through longitudinal studies, including patient follow-up and repeat testing, to guide its clinical application appropriately.

CHAPTER 3: APPLICATION OF ddPCR FOR DETECTION OF THE STAT5B^{N642H} MUTATION IN FELINE SCL AND CD4 T CELL LEUKEMIA

MATERIALS AND METHODS

Study population

Feline tissue biopsy samples submitted to Colorado State University Veterinary Diagnostic Laboratory for histopathology were collected. Feline blood samples submitted to Colorado State University Clinical Hematopathology (CSU-CH (Colorado State University Clinical Hematopathology)) laboratory for immunophenotyping by flow cytometry were also collected. All cats included in this study were older than 6 years of age. Two cohorts of patients were examined. The first cohort of cats were those with confirmed small cell T cell epitheliotropic lymphoma (SCL). The biopsy samples of these cats were evaluated by two board-certified anatomic pathologists, both blinded to the other's report. The second cohort of cats were those with confirmed CD4 T cell leukemia diagnosed by flow cytometry. PCR for Antigen Receptor Rearrangement (PARR) was performed on all samples.

Genomic DNA (gDNA) was extracted using a QIAamp DNA Mini Kit (QIAGEN according to the manufacturer's instructions) and DNA quantification was performed using a Nanodrop One (Thermo Fisher: ND-ONE-W).

Flow Cytometry

Data was collected retrospectively from CSU-CH laboratory database. Immunophenotyping by flow cytometry was performed on peripheral blood samples submitted to CSU-CH laboratory. Blood sample collection, storage and red cell lysis were performed as previously described. [27] Cell pellets were stained as previously described, using antibody solutions provided in Table 3. Propidium iodide staining was used to exclude dead cells during

analysis. All samples were analyzed on a 3-laser Coulter Gallios flow cytometer. Data analysis was carried out with Kaluza software (Beckman Coulter, Brea, CA). Total cell counts for CD4+ T cells were determined by multiplying the percentage within the gated lymphocyte population by the total lymphocyte count on the patient's CBC. Cell counts were compared to internally generated reference intervals. The upper limit for feline blood CD4+ T cells is 3,300/uL. [29]

Table 3. Antibody panel used for flow cytometric immunophenotyping.

Tube	Antibody specificity, fluorochrome and clone
1	None
2	CD4-FITC (vpg34) / CD8-PE (vpg9) / CD18-Alexa 647 (CA1.4E9)
3	CD5-FITC (FE1.1B11) / CD21-PE (CA2.1D6) / CD18-Alexa 647 (CA1.4E9)

All antibodies were purchased from Bio-Rad, Hercules, CA.
Each antibody tube (25 µL) was combined with 25 µL sample in a single well of a 96-well plate

Histopathology

Formal-fixed intestinal biopsy tissues were paraffin-embedded (FFPE) and stained with hematoxylin & eosin (H&E) for histopathologic examination. All cases were evaluated by two board-certified anatomic pathologists. To prevent observer bias, slide interpretations were performed blindly by both pathologists. A diagnosis of SCL was based on infiltration of a dense monomorphic population of small-sized lymphocytes within the small intestinal epithelium and lamina propria. Occasionally cases were described as causing architecture distortion and villous fusion, and the presence of intraepithelial lymphocytes forming plaques or nests.

PCR for antigen receptor rearrangements (PARR)

PARR was performed on all DNA samples extracted from both peripheral blood (CD4 T cell leukemia cats) and FFPE tissue (from SCL cats) to assess the clonality of T-cell receptor

gamma (TRG) gene rearrangements. PARR was performed as previously described.[27] (Rout et al., 2019). Inclusion criteria were cases in which there was a confirmed clonal TRG result by PARR.

Droplet Digital PCR

DdPCR reactions were performed as described in Chapter 2 material and methods for each patient. To evaluate the fractional abundance of the STAT5B^{N642H} mutation in the samples, assay thresholds were set based on positive and negative standards for each run (as previously described). The fractional abundance of the mutant allele was obtained by dividing the total number of copies per microliter of mutant alleles (FAM+, A) by the total copies per microliter of wild-type allele (HEX+; B) plus mutant: [A/(A + B)]. The determination of the number of target copies per droplet (number of copies of target molecule) was adjusted by the software to fit a Poisson distribution model with a 95% confidence level.

RESULTS

STAT5B mutation detection by ddPCR

Detailed results for each patient cohort are listed in Table 4. 27 cats met the inclusion criteria for the SCL cohort. All cats were definitively diagnosed with SCL by two board-certified anatomic pathologists, and all had clonal TCR rearrangements by PARR. Within this cohort, 66.7% (18/27) of patients were positive for the STAT5B^{N642H} mutation by ddPCR. 44 cats met the inclusion criteria for the CD4 T cell leukemia cohort. All cats were diagnosed with CD4 T cell leukemia by flow cytometry, and all had clonal TCR rearrangements by PARR. Within this cohort, 47% (21/44) were positive for the STAT5B^{N642H} mutation by ddPCR.

Table 4. Clinical features of cats.

Characteristics	Small cell T cell epitheliotropic intestinal lymphoma (SCL) cohort (N=27)	CD4 T Leukemia cohort (N=44)
Age (median, years)	12.8 (7-18)	11.5 (3-16.8)
Sex		
MC	13	20
FS	14	24
Breed		
Mix breed (DSH, DMH, DLH)	24	41
Purebred (ASH, MCC, SPHX, PERS, SIAM, SAV, RUSSB, RAG, CAL, NORFOR)	3	3
Average total lymphocyte count	-	30,680 (6,800 -263,500)
Average CD4 T lymphocyte count	-	23,805 (6,000-260,399)
PARR (TCR)		
Polyclonal	0	0
Clonal	27	44
STAT5B mutation status		
Present	66.7% (18/27)	47.7% (21/44)
Absent	33.3% (9/27)	52.3% (23)

Abbreviations: PARR, PCR for antigen receptor rearrangements; FI, female intact; FS, female spayed; MC, male castrated. Breed abbreviations: ASH, American Shorthair; CAL, Calico; DLH, Domestic Longhair; DSH, Domestic Shorthair; DMH, Domestic Medium Hair; MCC, Maine Coon Cat; MIX, Mixed Breed; NORFOR; Norwegian Forest; PERS, Persian; RAG, Ragdoll; RUSSB, Russian Blue; SAV, Savannah; SIAM, Siamese; SPHX, Sphynx

Fractional abundance of mutated STAT5B

The fractional abundance percentage was evaluated in samples where the STAT5B^{N642H} mutation was determined "present" by ddPCR (Table 5). There were 18 cats from the SCL group, 21 cats from the CD4 leukemia group, and 2 cats from the enteritis group in Chapter 2. The mean fractional abundance for the SCL group was 9.55% (range 0.18-34.6%). The mean fractional abundance for the CD4 T cell leukemia group was 34.88% (range 0.01-78.2%). The fractional abundance values for the enteritis cats were 0.13% and 0.09%. The fractional abundance values were plotted on a scatter plot for each cohort (Figure 7).

A simple linear regression was performed which showed there was no correlation observed between the fractional abundance and total CD4 T cell count in the CD4 T cell leukemia cohort (Figure 8).

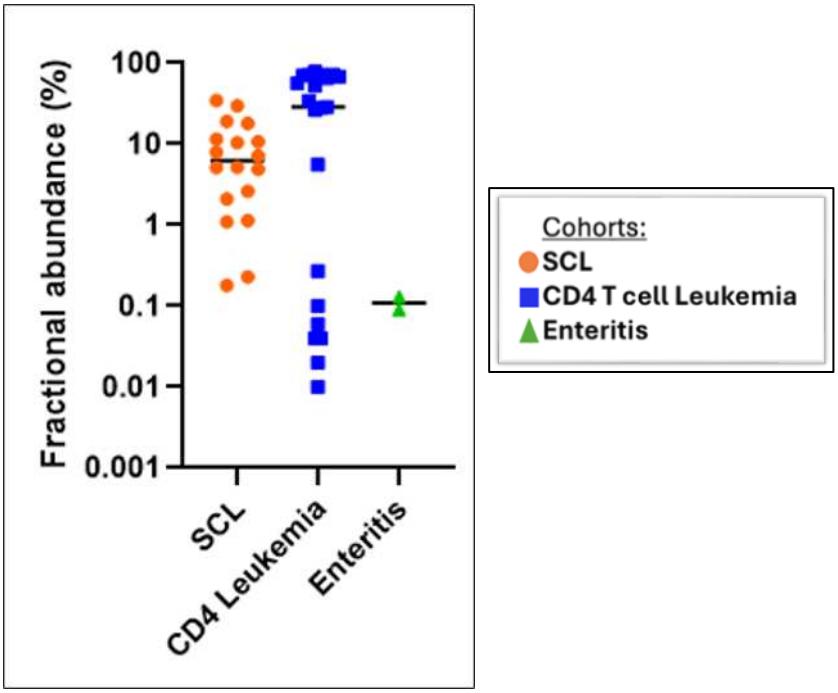


Figure 7: Scatter plots with fractional abundance (%) compared in each cohort.
The fractional abundance percentage (y-axis); Patient cohorts (x-axis). Each symbol is representative of a patient in each cohort in which the STATB^{N642H} mutation was detected by ddPCR.

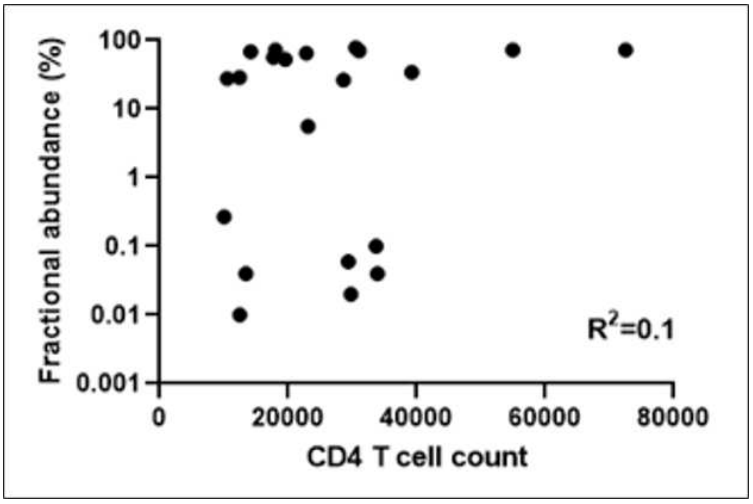


Figure 8. Simple linear regression demonstrating the correlation between the Fractional Abundance (y-axis) and total CD4 T cell count (x-axis) in the CD4 T cell leukemia cohort.

Table 5. Fractional abundance percentage (FA%) for each patient in each cohort (SCL and CD4 T cell leukemia), in ascending value.

Patient	**SCL Group, FA %**	**Patient**	**CD4 T cell leukemia group, FA%**
1	0.18	1	0.01
2	0.23	2	0.02
3	1.1	3	0.04
4	1.14	4	0.04
5	2.1	5	0.06
6	2.62	6	0.1
7	4.88	7	0.27
8	5.12	8	5.6
9	5.19	9	26.4
10	7.2	10	27.9
11	8	11	28.8
12	10.4	12	34.4
13	10.7	13	52.6
14	11.5	14	56.5
15	18.1	15	65.1
16	19.16	16	68.2
17	29.72	17	70.6
18	34.64	18	72.3
-	-	19	72.6
-	-	20	72.8
-	-	21	78.2

DISCUSSION

In this study, our primary objective was to apply a sensitive droplet digital PCR (ddPCR) assay targeting the STAT5B gene to discover the prevalence of the STAT5B^{N642H} mutation in feline patients diagnosed with small cell T cell epitheliotropic lymphoma (SCL) and CD4 T cell leukemia. The identification of this mutation holds significant clinical implications, as it may provide insights into disease pathogenesis and potentially guide therapeutic approaches. The group of cats diagnosed with SCL underwent a thorough diagnostic assessment, which was confirmed by histopathological examination independently conducted by two board-certified anatomic pathologists. Moreover, these cases showed evidence of clonal T-cell receptor (TCR) rearrangements, as verified through Polymerase Chain Reaction for Antigen Receptor Rearrangement (PARR). 27 cats met these criteria for inclusion. Subsequently, ddPCR analysis was conducted on all 27 samples, revealing that 66.7% (18/27) of these cats tested positive for the STAT5B^{N642H} mutation. In parallel, cats diagnosed with CD4 T cell leukemia were identified through flow cytometry analysis, all of which exhibited clonal TCR rearrangements confirmed via PARR. A cohort comprising 44 cats meeting these inclusion criteria underwent ddPCR analysis. The ddPCR results showed that 47% (21/44) of these cats were positive for the STAT5B^{N642H} mutation. This data demonstrates the high prevalence of the STAT5B^{N642H} mutation in both SCL and CD4 T cell leukemia diagnosed in cats.

These findings are consistent with what has been previously reported, with Kieslinger et al 2021 detecting the same STAT5B^{N642H} mutation in 29.41% (5/17) cats with SCL with a combination of Sanger Sequencing and ARMSqPCR. The greater percentage of cases detected in our study could be attributed to varied factors. As previously discussed, SCL can often be challenging to diagnose, and to determine the best representative cohort, the histopathology

biopsy reports were evaluated by two separate anatomic pathologists, both blinded to the other's diagnosis, to increase accuracy. Additionally, our study also performed PARR to confirm clonal T cell populations which also helped increase the accuracy in diagnosis. With that said, the most impactful diagnostic tool to increase the sensitivity of mutation detection was the development of the ddPCR assay, which has been reported to have increased sensitivity of mutation detection compared to other methods.[54–56,60,62] Importantly, our study demonstrated that the STAT5B^{N642H} mutation is not exclusive to SCL, as it was also detected in cats with CD4 T cell leukemia. This is comparable to what is found in human medicine, with the STAT5B^{N642H} mutation detected in many diverse types of human T cell lymphoma and leukemias. [30,31,35,37,49] The STAT5B^{N642H} mutation stands out as the most prevalent mutation identified in human primary intestinal T-cell lymphomas (ITCL). ITCL is comprised of enteropathy-associated T-cell lymphomas (EATL) and is further subdivided into EATL type I (associated with celiac disease) and monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL).[63] Although the STAT5B^{N642H} mutation is the most prevalent, it is not the sole mutation present in ITCL cases. Additional recurrent mutations found in human intestinal lymphoma cases encompass JAK kinases (JAK 1/3), components of the MAP kinase pathway such as BRAF, NRAS, and KRAS (G12A, G13D), chromatin modifier genes like SETD2 and CREBBP, and a G-protein-coupled receptor gene, GNAI2. [36,38,51,64] Exploring whether these recurrent mutations observed in human cases are also present in feline lymphomas and leukemias could offer valuable insights for future studies. Cats might be a suitable model for indolent digestive T-cell lymphoproliferative disorder of the gastrointestinal tract (iTLPD-GI) in humans. iTLPD-GI is a rare disorder characterized by superficial monoclonal intestinal T-cell infiltration and the disease is indolent or slowly progressive.[65–68] The etiopathogenesis of iTLPD-GI is not clearly understood but has been

reported to have overlapping features of inflammatory disease and aggressive intestinal lymphomas, suggesting potential shared disease mechanisms. [65,66,68–71] Mutations in the JAK/STAT pathway have been reported in iTLP-GI cases, however, no cases have yet to detect the STAT5B^{N642H} mutation that is found in MEITL. [67] Therefore, it is challenging to discern if SCL in cats is a more representative model of MEITL or iTLP-GI, or potentially both.

As a quantitative diagnostic test, the ddPCR assay can calculate the fractional abundance (mutant allele frequency) of the STAT5B^{N642H} mutation in each positive sample. This value is calculated based on the number of droplets analyzed in each sample and evaluates the number of mutant STAT5B copies divided by the total number of STAT5B copies screened (mutant and wild type). The wide spectrum of fractional abundance percentages observed among STAT5B-positive patients in both the SCL and CD4 T cell leukemia cohorts presents an intriguing discovery, raising questions about the variability in tumor burden and disease pathogenesis. Tumor burden can be detected and used to assess relapse or progression of disease after patients have undergone treatment. However, this can often be hard to detect in animal cancer patients, without advanced imaging, which can be costly and time-consuming. In human medicine, liquid biopsy samples have received favor over traditional biopsy samples, as these can potentially be less costly, time-consuming, and invasive for the patient.[72–74] Examples of liquid biopsy samples include the collection of patient blood, urine, or saliva to detect circulating tumor cells or circulating tumor DNA (ctDNA). Mutation detection in these samples could be performed using PCR-based methods, such as ddPCR. In human medicine, one group utilized ddPCR to detect BRAF/NRAS mutations in patients with metastatic malignant melanoma.[57] This group found that patients with circulating tumor DNA (ctDNA) at a fractional abundance below 1% at the start of treatment had significantly longer progression-free survival (PFS) ($p = 0.019$) and overall

survival (OS) ($p = 0.026$) compared to patients with higher ctDNA (fractional abundance >1%).[57] Additionally, patients with ctDNA levels receding fractional abundance below 1% during the treatment period had significantly longer PFS ($p = 0.002$) and OS ($p = 0.002$) compared to patients with stable or increasing fractional abundances of ctDNA. [57] In correlating this to feline medicine, a cat diagnosed with CD4 T cell leukemia with the STAT5B^{N642H} mutation detected by ddPCR could have serial ddPCR assays to assess the fractional abundance before, during, and after chemotherapy to assess patient response to treatment. This information may also provide better clinical practice guidelines for dosage and length of treatment standards for cats. This fractional abundance percentage could also offer valuable insights into a patient's disease risk. Further investigations, particularly outcome studies incorporating sequential fractional abundance measurements, are essential to establish potential threshold values with clinical significance. Such values could serve as pivotal markers for disease monitoring.

The CD4 T cell leukemia cats all had flow cytometry performed on their samples, which provides both a total lymphocyte count and a CD4+ lymphocyte count. Therefore, with this cohort of cats, we could compare the calculated fractional abundance from the BioRad droplet generator with the expected fractional abundance (calculated from the flow cytometry results) to determine whether these cats have a homozygous or heterozygous genotype for the STAT5B^{N642H} mutation. In considering the genotypic makeup of these cats, it is important to consider the concept of loss of heterozygosity, which can occur during cancer development and occurs in two forms.[75,76] One form is copy number loss, in which tumor cells will lose one allele because of partial chromosome deletion. The second form is copy number neutral loss, in which copy number loss could further undergo recombination using the homolog as a template; therefore there is no change in copy number.[75,76] Copy number neutral loss of heterozygosity has

been reported in human MEITL cases with mutated STATB. [36,41] This process leads to duplication of a maternal or parental chromosome or chromosomal region and concurrent loss of the other allele. In patients with MEITL, the mutated STAT5B allele is preserved and duplicated, and the wild-type allele is lost, which may give the impression that this mutation is homozygous. The preservation of the mutated STAT5B allele suggests the importance of this mutation in development of MEITL. If cats also undergo a similar loss of heterozygosity, then by ddPCR the fractional abundance for mutant STATB would theoretically be 100%, demonstrating an apparent homozygous genotype. However, loss of heterozygosity may not always be a distinct feature of this mutation in cats, as our data suggests by the variable fractional abundance observed amongst the different cohorts. Future studies to consider could include whole-exome sequencing to determine if this process occurs in lymphoma and leukemia diagnosed in cats, as it may indicate an important role in feline tumorigenesis.

Given the remarkable sensitivity of the ddPCR assay in detecting rare mutations, the diagnostic landscape extends beyond mere mutation presence or absence. Instead, it encompasses the presence of mutations, the proportion of mutant alleles, supplementary diagnostic data, and the patient's clinical presentation. This holistic approach provides a comprehensive understanding of the patient's diagnosis and underscores the importance of integrating various diagnostic parameters for effective patient management. Despite SCL being a common disease diagnosed in the feline population, there is still much to learn about the underlying pathogenesis and potential spectrum of disease amongst patients. These findings prompt further investigation into the molecular mechanisms underlying feline lymphomagenesis and the therapeutic implications of targeting the JAK/STAT pathway in affected individuals.

CHAPTER 4: CONCLUSIONS

Our study demonstrated that our ddPCR assay can accurately detect and distinguish the wild-type and mutated STAT5B transcription factor with high sensitivity in cats' gDNA samples. Our assay showed that young cats without a diagnosis of lymphoid neoplasia rarely carry the $STAT5B^{N642H}$ mutation. Additionally, our assay demonstrated that cats with small cell T-cell epitheliotropic intestinal lymphoma (SCL) and CD4 T-cell leukemia frequently carry the $STAT5^{N642H}$ mutation.

These findings suggest that the $STAT5B^{N642H}$ mutation plays a role in the pathogenesis of lymphoma and leukemia development in cats, comparable to what has been reported in human lymphoproliferative neoplasms. Two young cats were diagnosed with enteritis (i.e. inflammatory bowel disease) and that had polyclonal TCRs by PARR had detectable $STAT5B^{N642H}$ mutations by our ddPCR assay. Upon follow-up investigation, both cats were reported to be clinically healthy after their diagnosis and initiation of immunosuppressive therapy (Prednisolone) and supportive care, with no change in diagnosis to SCL. However, SCL in cats is often reported to have an indolent disease course, and it has been hypothesized that cats with inflammatory bowel disease (IBD) can progress to developing SCL, although this has yet to be reported in the literature. The hypothesis of IBD progressing into SCL is presumably multifactorial in origin and depends on genetics, environmental factors, and the overall health status of the patient. It is also unclear what role early immunosuppressive therapy treatment, as both young cats in this study received, could play in disease progression and development. Additionally, given that the $STAT5B^{N642H}$ mutation was not detected in all the SCL and CD4 T-cell leukemia patients in our study, it is suggestive that other oncogenic mutations play a role in tumorigenesis, similar to what has been reported in human medicine. Further investigation through advanced molecular

diagnostics such as RNA sequencing or whole-exome sequencing is warranted to further characterize the genetic landscape in cats with lymphoma/leukemias.

Our ddPCR assay also showed that cats with the STAT5B^{N642H} mutation had a wide range of mutational fractional abundance. In human medicine, fractional abundance has been evaluated as a measure of patient survival and response to treatment. Patients with a higher fractional abundance have been shown to have decreased overall survival times and poorer responses to treatments for their respective cancers. Monitoring the STAT5B^{N642H} fractional abundance by ddPCR in cats confirmed with SCL and CD4 T-cell leukemia in larger outcome studies is warranted. This information could be used to compare a patient's response to treatment protocols and evaluate novel treatments. The current options for chemotherapy treatment in cats are limited. With expanded knowledge of the genetic landscape of tumor cells, additional treatments such as JAK/STAT pathway inhibitors could be utilized for more targeted therapies. STAT5B mutations are associated with increased phosphorylated protein and growth advantage. Therefore, these inhibitors may be more effective in patients with JAK/STAT pathway mutations, such as the STAT5B^{N642H} mutation detected in feline SCL and CD4 T-cell leukemias. These targeted therapies should be explored to enhance treatment and patient care in cats with lymphomas/leukemias.

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