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Lymphoproliferative disease in cats and dogs: a comparison of immunophenotyping by flow cytometry and clonality testing.

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CICLO XXXI

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CHAPTER 5- FELINE CLONALITY ASSAY.

Abstract

This thesis is a retrospective study on canine and feline lymphoproliferative diseases advanced diagnostic techniques. In particular, we focus our attention on immunophenotyping by Flow Cytometry (FC) and clonality assays, comparing the diagnostic sensitivity and specificity of the latter in the course of canine leukaemias and feline lymphoproliferative diseases.

In Chapter 1, an overview of lymphoproliferative diseases was given: the importance of immunophenotyping in the classification of such diseases, and its prognostic value have been well established in the last decade of research. However, immunophenotype is still unable to distinguish in some cases between reactive and neoplastic disorders, making clonality assays a useful, complementary tool in the diagnostic process. PCR for antigen receptor rearrangement (PARR) is a molecular biology technique aimed to amplify receptor genes which are clonally rearranged in the course of a clonal neoplastic expansion. Due to the potential application of PARR to many kinds of source material and due to the significant advantages offered by FC as a secure, cost and time effective technique, we believe that FC and PARR could be a valid alternative to the more expensive and complicated biopsy with histology and immunohistochemistry, which to date is considered the gold standard in lymphoproliferative diseases diagnostics.

In Chapter 2, we optimised the protocols for extraction of sufficient DNA from our samples: due to the retrospective nature of our study, we believed that having optimised protocols using the minimum volumes of source material possible with the best performances, could be useful for our purposes. We optimised protocols for whole EDTA blood (used in Chapter 4 for the canine clonality assays) and cytological slides (used in Chapter 5 for the feline clonality assay). The first experiments on blood were performed using two kits (GenElute Mammalian Genomic DNA Miniprep Kit by Sigma Aldrich and the QIAMP Mini and Blood kit from Qiagen): we selected the Sigma kit protocol using as source material sample volumes containing a white blood cell count (WBC) of $9x10^6$/mL and optimising the elution step. Extraction from cytological slides was optimised combining three protocols and creating a new original one, with optimised cell lysis and elution steps. The protocols developed were used for further experiments in the succeeding chapters.

In Chapter 3 we established feline positive controls as internal controls for the following experiments run in Chapter 5. We selected four feline patients, diagnosed with lymphoma by histology and immunophenotyped by IHC. After extraction of DNA from FFPE tissue samples, we performed PARR using the primer set intended to be used in the following experiments in order to select the samples which were positive to the single primers. Due to the immunophenotype and the positive clonal amplification of the TCRG gene, one sample was selected as a positive control for T cell clonality. One other patient was selected, according to the same criterion, as positive control for B cell clonality testing. However, DNA obtained from two feline lymphoma cell lines (MS-4 and FT-1, for B and T cell clonality respectively) were used in the present study along with our established positive controls.

In Chapter 4 the value of PARR in comparison with FC in the course of canine leukaemias was investigated. Twenty-nine cases were selected: of those, nineteen were diagnosed with leukaemia, and ten showed a raised WBC attributable to infection or immune-stimulation. The first group comprised: eight dogs diagnosed with T-ALL (of which, one was showing evidence of LGL leukaemia), four AUL, three B-ALL, two biphenotypic leukaemias, one T-CLL and one AML. PARR was performed using two primers directed to the TCRG locus, and two directed to the IGH gene. Separation and visualisation of PCR products were performed by Agarose gel electrophoresis. Overall sensitivity and specificity of clonality testing using FC as gold standard were 73% and 100% respectively. Neoplasia was detected in approximately 63% of the neoplastic cases diagnosed by FC; all the non-neoplastic cases were confirmed as reactive by PARR. PARR confirmed phenotype in 50% of the cases of B and T cell neoplasia; moreover, two AUL was diagnosed as T cell neoplasia, and the double phenotypic leukaemias clonally rearranged just the TCRG locus. However neoplasia was not detected in high rates, and cell lineage did not match between the two techniques in many cases: for this reason, PARR should not be considered as a diagnostic tool by itself but has to be always integrated into a more comprehensive diagnostic process.

In Chapter 5, clonality testing was performed on feline lymphoma/leukaemia and reactive lymphoproliferative disorders samples. Of the thirty-seven cases retrieved from the Dick White Referrals (DWR) laboratory archive, only twenty-one cases provided sufficient amounts of good quality DNA from the cytological slides available. Thirteen cases were diagnosed as having neoplasia (seven T cell neoplasia and six B cell neoplasia), in the peripheral blood (four cases), affecting the gastrointestinal tract (four cases), or peripheral lymphnodes (three cases) or other locations (two cases). Five cases were diagnosed as having a reactive process. In three cases a clear-cut distinction between neoplasia and reactive hyperplasia was not possible by the FC analysis only. Clonality testing was performed using a primer set comprising one primer directed to the TCRG locus and five primers directed to the IGH gene. Agarose, PAGE and capillary electrophoresis (CE) were performed and compared. If DNA was available for the reactive cases, detection of IGH gene rearrangement was performed using additional primers. Clonality testing on our feline case series showed an overall sensitivity and specificity of 63.6% and 100% respectively, and neoplasia was detected in 42,5% of the cases of neoplasia diagnosed by FC. PAGE and CE showed a good concordance, but CE has to be preferred especially in ambiguous cases where a clonal population is present within a polyclonal background. Our results showed the potential of PARR in refining the diagnosis of lymphoma achieved by FC, but highlights as well the weakness of the technique in terms of low sensitivity, possibly due to incomplete gene coverage of the primers used. Moreover, it confirmed the importance of using high resolution and highly sensitive techniques for visualisation of PCR products, such as automated capillary electrophoresis.

Abbreviations.

A: Adenine.

A_{230}: Absorbance at 230nm wavelength.

$A_{260/230}$: A_{260}/ A_{230} ratio.

$A_{260/280}$: A_{260}/ A_{280} ratio.

A_{260}: Absorbance at 260nm wavelength.

A_{280}: Absorbance at 280nm wavelength.

AL: Alimentary Lymphoma.

ALL: Acute Lymphoblastic Leukemia.

AML: Acute Myeloid Leukaemia.

APC: Antigens Presenting Cells.

AR: Antigen Receptor.

AUL: Acute Undifferentiated Leukaemia.

B-CLL: B cell Chronic Lymphocytic Leukaemia

BCR: B-cell Receptor.

BSA: Bovine Serum Albumin.

Bp: Base Pair.

C-: Constant Region of the AR genes.

C: Cytosine.

CBC: Complete Blood Count.

CD: Cluster of Differentiation.

CDR: Complementary Determining Regions.

CE: Capillary Electrophoresis.

CKCS: Cavalier King Charles Spaniel**.**

CLL: Chronic Lymphocytic Leukemia.

CRP: C Reactive Protein.

D-:Diversity Region of the AR genes.

DLH: Domestic Long Hair.

DNA: Deoxyribonucleic Acid

dNTP: DeoxynucleotideTriphosphate

DSH: Domestic Short Hair.

DTT: Dithiothreitol.

EDTA: Ethylene Diamine Tetraacetic Acid.

Fab: Fragment Antigen binding region.

FAB: French American British.

fAR: feline Androgen Receptor.

FC: Flow Cytometry.

FELV: Feline Leukaemia Virus.

FFPE: Formalin Fixed Paraffine Embedded.

FNA: Fine Needle Aspiration.

FR: Framework Regions.

G: guanine.

gDNA: genomic DNA

Hb: Haemoglobin.

HCT: Haematocrit.

HRM: High Resolution Melting (analysis).

ICC: Immunocytochemistry.

Ig: Immunoglobulin.

IGH: Immunoglobulin Heavy chain.

IGHJ: J- region of the IGH.

IGH-V: V- region of the IGH.

IHC: Immunohistochemistry.

J-: Joining region of the AR genes.

JRT: Jack Russell Terrier.

LGL: Large Granular Lymphocytes.

LGL-CLL: Large Granular Lymphocyte- Chronic Lymphocytic Leukaemia.

LMP: low melting point.

MHC: Major Histocompatibility Complex.

MRD: Minimal Residual Disease.

NCIWF: National Cancer Institute Working Formulation.

NGS: Next Generation Sequencing.

NHL: Non-Hodgkin's Lymphoma.

NK: Natural Killer (cells).

NTC: Non-Template negative Control.

ORF: Open Reading Frame.

PAGE: Polyacrylamide Gel Electrophoresis.

PARR: PCR for Antigen Receptor Rearrangement.

PBS: Phosphate-buffered saline solution.

PCR: Polymerase Chain Reaction.

RBC: Red Blood Cell Count.

REAL: Revised European American Lymphoma (classification).

RES: Reticuloendothelial System.

RNA: Ribonucleic Acid.

RQ-PCR: Real-time Quantitative PCR.

RT: Room Temperature.

SB: Southern Blot (analysis).

SDS: Sodium Dodecyl Sulfate.

SHM: Somatic Hypermutation.

T: Thymine

TAE: Tris-Acetate EDTA (buffer).

T-ALL: T cell Acute Lymphocytic Leukaemia.

TBE: Tris-Borate EDTA (buffer).

T-CLL: T cell Chronic Lymphocytic Leukaemia.

TCR: T cell receptors.

TCRG: locus of the TCR gene econding for the γ chain.

UV: Ultra-Violet.

V-: Variable region of the AR genes.

WBC: White Blood Cell Count.

WHO: World Health Organization.

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CHAPTER 1:

INTRODUCTION

Lymphoid proliferative diseases are described as a subset of conditions affecting the reticuloendothelial system (RES)[1]. The distinction between malignant proliferation and benign reactive processes is crucial: in the first event, the uncontrolled proliferation is driven by mutations which evade the cell lifespan control mechanism; the second condition is consequent to immune stimulation, caused by external agents (bacterial, viral, parasitic, allergenic agents) or auto-immune stimuli.

1.1 HEMATOPOIETIC MALIGNANCIES.

A general valid distinction between hematopoietic malignancies is that "*malignancy in a largely undifferentiated lymphocyte is likely to occur in the marrow of a young individual and present as leukaemia,* and *malignancy in a mature lymphocyte will likely occur in the peripheral tissues of a mature individual and present as lymphoma."*[1]. In fact, if one considers bone marrow as the most affected organ, with the presence of >20% of malignant cells, the diagnosis of Acute Lymphoblastic Leukaemia (ALL) can be achieved; Moreover, Chronic Lymphocytic Leukaemia (CLL) is defined as the presence of small mature lymphocytes in high numbers in the peripheral blood, making leukaemias a disease of bone marrow and blood. Conversely, lymphoma is characterised by the proliferation of malignant cells mostly present in the lymph nodes and/or other tissues. However this distinction is not so sharp: for example, ALL can involve secondary hematopoietic organs; or in the case of both Stage V lymphoma and CLL, circulating malignant cells can be detected, and secondary involvement of lymph nodes and/ or spleen can be present, making the distinction challenging[1].

In general, lymphomas represent a diverse group of diseases, which vary in microscopic appearance (architecture, cellular morphology) and behaviour, with a different rate of dissemination and progression and the potential for involvement of any location.

Lymphomas are amongst the most common types of tumours in dogs and cats[2]. There are remarkable similarities between the clinical features of canine lymphomas and human Non-Hodgkin's Lymphoma (NHL)[3]. The disease can be classified according to the anatomical localisation of the neoplastic cells; multicentric, alimentary, mediastinal,

or extranodal forms have been recognized in both dogs and cats, but the anatomical distribution seems to follow different rules in the two species. The most common type in the dogs is the multicentric[2], but this localisation appears to be rarer in the feline counterpart. In fact, it has been reported that Alimentary lymphoma (AL) is the most common form in the cat[4–7], especially in the post FeLV infection and current vaccination era[8]. Clinical staging of lymphoma accounts for five different stages depending on the localisation of the neoplasia and two substages according to clinical presentation[9]. Stage[10] and substage have been found to be directly linked to survival in the cat[6,11].

Classification criteria for lymphomas rely on human guidelines because lymphoproliferative diseases in animals tend to mimic in appearance and behaviour the human counterpart[12]. Therefore human classifications have been applied in Veterinary Medicine. Consensus guidelines for diagnosis and classification are now mandatory in Veterinary Medicine to assist the best management of the disease and provide prognostic information.

Different classification schemes have been successfully applied to feline and canine lymphoproliferative diseases[13]. The Kiel classification (first proposed in 1974, then revised in the Updated Kiel classification in 1988 and 1990)[14] has been successfully adapted to feline and canine lymphoid cancers[15,16] and distinguishes subtypes according to the immunophenotype of the cells and the grade (low/high), correlating cell morphology. More recently, the Revised European American Lymphoma (REAL) and the World Health Organization (WHO) classifications have been established. They both combine tumour cell morphology, immunophenotype, genetic features and clinical manifestations, but they do not classify the neoplasia through the high/low-grade approach[13]. Moreover, the WHO classification covers all the hematopoietic malignancies regardless of their origin. These classifications have been both successfully applied to companion animals[7,17]. Another classification scheme is the National Cancer Institute Working Formulation (NCIWF)[18–21], which is based on histopathologic features and identifies three subgroups (low, intermediate and high grade); it appears to be predictive of the biological behaviour. Furthermore,e the “French American British” (FAB) scheme

has been used in Human Medicine[22,23] and Veterinary Medicine[24,25] for classification of acute leukaemias, which distinguishes lymphoid and myeloid lineages not only according to cell morphology but also according to immunological features.

1.2. DIAGNOSIS OF LYMPHOPROLIFERATIVE DISORDERS.

1.2.1. ROUTINE LYMPHOMA DIAGNOSTICS.

While histopathological analysis and immunohistochemistry have been suggested to be the gold standard diagnostic tools in such malignancies[26–28], cytology is often used as a fast, cost-effective tool to aid the diagnosis of lymphoma. Histology has the great advantage of giving the architecture of the lesion and the degree of cell proliferation and surrounding tissue invasion. Nevertheless, the acquisition of tissue samples, other than being an expensive and invasive procedure requiring general anaesthesia, can be difficult especially for some anatomical forms such as intestinal lymphoma, where biopsies are mostly taken endoscopically and often limited to the mucosa. Such specimens are often not sufficient to give a complete picture of the disease. Moreover, preparation of tissue samples requires longer times and more equipment than cytology, which is an easy technique performable even in small practices. Cytology, however, has its drawbacks, with a high risk of missing the neoplastic cells within a vast lesion and not giving any information on the architecture of the lesion. Moreover, despite the pathologist's or clinical pathologist's experience, identification of neoplastic cells (especially for indolent diseases, early stages of disease or presence of a residual population of normal lymphocytes) can be challenging. In addition, a morphological assessment with routine staining techniques only is nowadays considered insufficient to detect the cell lineage, especially if the distinction between myeloid vs lymphoid diseases is required.

However, cytology is considered sufficient for a first assessment of non-Hodgkin's lymphomas, and it is considered a valid diagnostic technique especially if coupled with immunophenotyping by flow cytometry[29–31]. Its usefulness has also been shown in veterinary medicine for refining lymphoma diagnosis[32,33].

1.2.2. IMMUNOPHENOTYPING.

Most of the classification schemes in use nowadays in Veterinary Medicine, consider the cell lineage as a discriminatory criterion between subtypes. The classifications nowadays applied to companion animals lymphoid leukaemias, in fact, distinguish between B and T cell diseases, and seems that phenotype, together with other variables, such as the anatomical location, histological and clinical grade, is related to survival time[34]. Moreover, FAB classification applied to Veterinary myeloid leukaemias distinguishes the different entities by cellular origin.

Immunophenotyping allows the detection of polypeptides ordinarily present on cell membranes or in the cytoplasm, the so-called "cluster of differentiation" (CD), by the use of a panel of monoclonal antibodies, which selectively label these proteins. If the pattern of expression is homogeneous within the lesion, a diagnosis of neoplasia can be achieved. Expression of a specific CD or of a combination of more CDs by the cells allows the recognition of the cell lineage. In the course of neoplasia, cells can increase or lose CD expression, or show aberrant patterns[35]. Any type of source material can be virtually submitted to immunophenotyping: tissue samples (immunohistochemistry, IHC), cytological preparations (immunocytochemistry, ICC), or cell suspensions (flow cytometry- FC).

The first two techniques (IHC and ICC) show a limited antibodies availability. Routine differentiation between B and T cell malignancies, in fact, is made upon positivity to CD79a and CD3 respectively[21,36]. Flow cytometry indeed offers a more extensive antibody panel, allowing better characterisation of the neoplasia[37,38] than cytology or histology, even if combined with ICC or IHC respectively. For example, a routine diagnostic panel for leukaemias in dogs using FC includes from 12 -17 mAbs (Table 8).

Moreover, FC allows to combine information about immunophenotype and morphological cellular features, such as size and cytoplasmic complexity, providing precise identification of the cell line. In particular, the hallmarks of FC in lymphoproliferative diseases are 1) detection of clonality which enables identification of

the ongoing neoplastic process 2) identification of lineage or lineage infidelity, possibly with higher sensitivity than IHC, given the broader panel of antibodies 3) detection of maturation stage and consequently biological behaviour. It has been described how cells with "blastic" features (such as expression of the marker CD34) tend to have higher proliferation rates and more aggressive behaviours[39–41].

Cytometers have traditionally been used to define different cell populations in blood, based on light scatter properties: flow cytometry adds to the ability to measure the physical characteristic of the cells, the detection of CD expression pattern by the use of fluorescently labelled antibodies. In fact, the cells examined are redistributed into a laminar flow and are individually passed through a laser beam. The light scattered forward and the light scattered on the sides of the cell allows determination of the size and the internal complexity of the cell respectively. Moreover, the fluorescent labels (fluorochromes) bound to the antibodies, are excited by the light beam, and detectors collect emitted light and then digitally converted. The use of different fluorochromes with similar excitation wavelengths but different emission wavelengths enables the simultaneous detection of the various CDs, giving a better characterisation of the phenotype.

Combined information about size, cytoplasmic complexity and CD pattern expression, detected homogeneously in the sample, aids the diagnosis of lymphoma/ leukaemia, which cannot, in any circumstances be used alone, disregarding clinical information and morphological assessment by cytology.

However, Flow Cytometry is an easy and fast procedure which provides cost-effective results in a couple of hours. It applies to any cell suspension, from blood and cavitary fluids to FNA samples, which can be processed after stabilisation in medium (usually buffered culture medium with serum or albumin[37]). A minimum number of 2 million cells have to be contained in the sample to achieve good results and application of a complete panel of antibodies. Moreover, the specimen has to be fresh (possibly analysed within 48h from collection[33,42]), thus representing one of the limitations of the technique, as fast and correct shipment of samples cannot always be possible.

In the dog, immunophenotype has been demonstrated to be a significant prognostic factor. Despite B cell lymphomas being reported as having a better prognosis[20,43–46], low-grade T cell lymphomas are considered to be mostly indolent; high-grade T cell lymphomas are indeed the most aggressive with short survival times[45]. The opposite results have been shown in the course of chronic lymphocytic leukaemia, with T-CLL having a better course than B-CLL, but atypical CLL has an aggressive behaviour with a poorer prognosis. The role of immunophenotype in acute leukaemias has not been demonstrated in the canine species[47]. However other contributors were involved in survival prediction. In the dog extranodal forms of lymphoma seem to have longer survivals and a less aggressive course than the multicentric type[48]. This is true also for the cat patients, with alimentary and renal lymphomas carrying the worst prognosis[49–52]. In one study on the cat, T small cell lymphomas were proven to be the most common type of treated alimentary lymphoma, with longer survival and good remission rates[53]. Nonetheless, a clear correlation between phenotype and prognosis in felines has not been proven to date[10,34,52,54], probably due to the lack of antibodies available and validated for this species.

1.2.3. CLONALITY ASSAYS.

In the case of indolent forms of lymphomas or profound reactive hyperplasia, the distinction between reactive and neoplastic conditions is not so evident. Some features observed in the course of Large Granular Lymphocytes (LGL) leukaemia, for example, can overlap some other findings during infectious diseases[55]. Moreover, inflammatory bowel disease and intestinal lymphomas (especially the indolent forms), can be virtually indistinguishable by cytology and histology, even if assisted by immunophenotyping.[56]

Clonality is a hallmark of neoplasia: according to the somatic mutation theory of carcinogenesis, spontaneous mutations are responsible for tumour development and are clonally transmitted during malignant proliferation. The detection of a genetically homogeneous population is the principle of clonality testing and the distinguishing criterion between reactive and neoplastic lesions.

In lymphoproliferative disorders, the targets of clonality detection are the Antigen Receptors (AR) genes: these genes, under physiologic conditions, drive the synthesis of the membrane-bound ARs: Immunoglobulins (Ig) on the surface of B lymphocytes (chief representatives and effectors of humoral immune response) and T cell receptors (TCR), expressed by T lymphocytes (a significant component of cell-mediated immunity). The ARs are highly diverse within a normal lymphocyte population: this fact assures the ability of recognition of a wide variety of antigens. Diversity in Ig or TCR structure is estimated to be in the order of 10^{12}, making the event of two lymphocytes carrying the same receptor very unlikely[57]. The high genetic diversity is assured by the recombination and random joining of the regions composing the Ig and TCR genes, namely the Variable (V), Joining (J) constant (C) and Diversity (D) regions. In the first instance, the joining process starts with the D-J conjunction, followed by the attachment of the V region. For those genes not containing the D sequence, the process involves VJ joining only. The last step is the (D)J-V joining to the C region. This mechanism potentially leads to a vast number of possible recombinations, which results in a wide receptor structure diversity, reflecting the high recognition and antigen-binding potential. Variety is also enhanced by random nucleotides insertion and deletion, as well as point mutations occurring between the joined regions. Moreover, some genes contain more than one C regions which can also undergo rearrangement. Additionally, antigenic stimulation provokes somatic hypermutation in B cell Receptor (BCR) genes, with insertion of point mutations all along the Immunoglobulin Heavy chain (IGH) gene.[58]

Conversely compared to a diverse, mixed lymphocyte population, a neoplastic population will show a restricted genetic pattern, given the fact that all the cells are identical clones of each other.

Clonality testing in Veterinary Medicine has been of interest in the last decade, representing an adjunctive tool in refining diagnosis of lymphoproliferative diseases, especially in the more controversial cases. These tests have been directed to amplification and detection of AR genes, and the molecular assays available vary in

design and complexity and include Southern blot (SB) analysis, Polymerase chain reaction (PCR), and real-time quantitative PCR (RQ-PCR).

Southern blot analysis has been used with success in a few reported studies in Veterinary Medicine[59,60]. Although there are excellent specificity and sensitivity of the assay, it presents many limitations, mainly technical. First of all, it is a time-consuming technique, requiring around seven days for sample processing and result interpretation; moreover, it needs fresh samples of good quality, precluding application to the fixed or archived material. Finally, it has a low analytical sensitivity, since it is unable to detect a small proportion of monoclonal cells mixed in an extensive background of normal polyclonal lymphocytes[61]. Finally, a significant limitation of the conventional Southern Blotting technique is its inability to detect gene rearrangements at much below the 5% level[62].

Conversely, the popularity of Polymerase Chain Reaction (PCR) in routine diagnostics has arisen due to the versatility of the technique. It can be applied to fresh, frozen or fixed tissue; to archived samples such as formalin fixed- paraffin embedded (FFPE) tissue or stained cytological slides; to fluids and cell pellets obtained from fine needle aspiration (FNA). DNA can be extracted from virtually any source matrix, making PCR for antigen receptor rearrangement (PARR) a useful and convenient technique to be used in the Veterinary diagnostics routine. After DNA extraction, the PCR-based amplification is performed using a set of primers, to detect and amplify the AR genes.

The amplified products are then separated by electrophoresis: the first studies reported Agarose gel electrophoresis as suitable for visualisation of prominent bands in the case of neoplasia, but this method has been replaced by higher resolution techniques such as polyacrylamide gel electrophoresis (PAGE). Nowadays, automated capillary electrophoresis (CE) is considered the best method for clonality detection[27], and interpretation of results has been standardised by the EuroClonality/ BIOMED-2 guidelines applied to Veterinary clonality assays[58,63]

To date, the primers designed in the dog and the cat are directed to the TCRG locus (encoding for the γ chain of the TCR) and the IGH locus (encoding for the Heavy chain of the membrane-bound immunoglobulin).

However, PARR is a sophisticated technique: it requires basic molecular biology and genetics knowledge, and special precautions are needed during sample processing and interpretation. This technique albeit useful and full of potential is not an easy technique and has many limitations.

First of all, incomplete gene coverage by the primers in use can occur: to enable the highest efficiency possible, many studies have been carried out in both canine and feline lymphoproliferative diseases. These studies provided the primers now routinely used in diagnostics.

In the dog, the first approaches to clonality assays date back to the last years of the nineties, when the pioneers Vernau and Moore (1999) designed primers directed to the canine TCRG locus[39]. Years later, Burnett e al.(2003), developed a new primer set for amplification of canine TCRG and Ig genes[64]: namely two primers for B cell (IgH major and IgH minor) and one for T cell clonality detection. Primer design was implemented after the publication of the canine genome[65,66] when more accurate sequences alignment was possible. New primers were designed by a Japanese research group[67,68], and other groups[69–71]. In the last few decades, Keller et al.(2012) have improved the assay, developing a multiplex PCR detecting a more extensive range of rearrangements for TCRG[72] even if more than one reorganisation per allele is present, resulting in increased sensitivity.

Knowledge of feline primers and clonality assays is more limited than in the canine counterpart. A first effort to define and detect the feline IGH gene was made in 2005 by Werner et al.[73] In this study analysis of twenty-four transcripts obtained from normal feline splenic lymphocytes allowed the description of the IgM locus and the design of primers for B cell clonality assessment. The target of the experiment was the Framework Region (FR) 2 and the FR3 of the IGH gene. Two consensus forward primers placed into

the V region (IGHV) and three antisense primers (in the J segment) were outlined. Nevertheless, no clan/family association could be assessed at that stage. Later on, Heinrich et al.[74] grouped feline IgH genes into two families (IGH1 and IGH3) and the sequences obtained by Werner some year before could be placed into the family IGH3. This study designed two pairs of primers directed to FR1 and FR3 of both gene families. The TCRG locus was widely described by Weiss et al.[75], and a primer set was designed[76]. Ultimately, the Japanese group of Mochizuki et al. obtained other sequences both for the feline IGH gene and the TCRG, designing a wider primer set than the existing ones[77,78], designed for multiplex reactions, thus improving sensitivity and specificity.

To date, feline PARR has a maximum sensitivity of 91% for TRG clonal rearrangement detection (ranging from 46% to 91% within three different published primer sets)[73,76,78] and of 89% (ranging from 64 to 89%)[74,77] in B cell clonality assays.

As a more extensive primer set is available for diagnostics and research in the canine species: B cell clonality assays have shown high sensitivity (ranging from 80% to 98%)[70,79] and specificity (99%)[80]. PARR has detected lymphomas/ leukaemias of T-cell lineage with a specificity of 86-98%[70,80]and a sensitivity improved to 100% in some studies[81], which make this method of interest to most clinical-pathology laboratories.

The variable sensitivity and specificity rates are due to a plethora of reasons, mainly involving the difficulties in primer design and complete gene coverage, as aforementioned, mainly due to the lack of knowledge in the genomic structure of TCR and Ig structure and encoding genes.

To date, the TCR has been described as a polypeptide bound to the surface of T lymphocytes. It is associated with CD3, forming the TCR-CD3 complex (**Figure 1**); it represents the primary effector of cell-mediated immune response, with its exclusive ability in recognising foreign molecules only if presented by antigen presenting cells (APC) through the major histocompatibility complex (MHC) molecules. The receptor is composed of four polypeptide chains, responsible for the αβ or the γδ phenotype.

In humans, dogs and cats the predominant phenotype of circulating T cells is αβ phenotype, whereas a small percentage expresses the γδ heterodimer[82]. The γδ lymphocytes have been reported to be present in skin, lungs, intestine and reproductive organs lining epithelium[83], to recognise antigen, not MHC bound and also to act as antigen presenting cells[65,83], representing the link between innate and adaptive immunity.

Each αβ or γδ chain is composed of a Variable domain (V), a constant domain (C), a transmembrane region and a cytoplasmatic termination. Each chain is encoded in a distinct locus within the chromosome (the α chain in TCRA locus, the β in TCRB, the γ in TCRG and the δ in TCRD). The locus comprises different segments which, in turn, encode for the single domains: thus, variable domain is encoded by the Variable (V-) segment, joined to the constant domain encoded by constant (C-) region by peptide encoded by Joining (J-) region. Diversity in some phenotypes is additionally enhanced by the presence of a Diversity (D) sequence (not present in TCRG locus). TCR structure is illustrated in **Figure 1**[82].

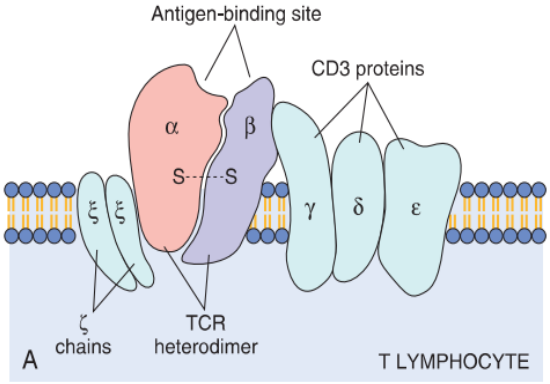


Figure 1. T cell receptor structure (αβ phenotype).

Despite the final phenotype, the first locus rearranged during T cell development is the TCRD, followed by TCRG locus. The lymphocyte can afterwards maintain the γδ phenotype or can proceed to TCRA rearrangement first, and consequently of TCRB locus, achieving the αβ phenotype[84]. Nonetheless, the TCRG is retained despite the phenotype, representing a reasonable target for the clonality assay. Moreover, the organisation of canine TCRG in multiple cassettes gives more chances for the designed primers to cover more than one of all the possible rearrangements[85]. For these reasons, TCRG is the primary target of PARR.

With an approximate extension of about 500kb, the canine TCRG gene is located on chromosome 18. The different regions are grouped in eight cassettes aligned in tandem and divided one from another by a 10-18 bp space (except the area between cassettes 6 and 7, which is slightly larger). A total of 40 regions (8 TCRG-C, 16 TCRG-V and 16 TCRG-J) are diversely combined throughout the loci and not all the genes are functional. The V domains (target of amplification in clonality assays) comprise three Complementarity-determining region (CDR) intercut with four FR, similar to IgV gene[86]. Eight out of sixteen V segments are pseudogenes: the remainder assigns to 4 subgroups: TCRV2 (comprising four genes), TCRV4 and TCRV5 with one gene each and TCRV-7 with two genes. Within the J regions, seven are functional while seven are pseudogenes and two are Open Reading Frames (ORF). Six out of eight constant genes are functional, whereas TCRC1 is an ORF and TCRC6 is a pseudogene. The canine TCRG locus is illustrated in **Figure 2**.

The feline TCRG gene is located on chromosome 8. It comprises, similar to the dog different V-, J-, C- regions, differently situated throughout the locus (**Figure 3**). Eight V-regions have been described so far: of these five seem to be functional and were divided into three subgroups: subgroup one consisted of three members (V1, V2, V3)[75,87], while subset 2 and 3 included one each (V4 and V5 respectively). the remaining three V sequences are likely pseudogenes and show high homology to the V4 sequences (V4.1P, V4.2P, V4.3P)[88]. J regions described so far have been attributed to three subgroups, with five members (J 1.1, J 1.2, J 1.3, J 1.4, J 1.5) in the first, two in the second (J2.1, J2.2)[88]

with one probably a pseudogene and one in the third (J3)[75]. An additional J region not attributable to any subgroup has been described, thus representing a pseudogene[75]. Finally, six feline C- regions of TCRG locus have been described (C 1, C 2, C 3, C4, C5, C6)[75].

Similar to the dog, each feline TCRG gene comprises three CDRs intercut with four FRs (**Figure 3**).

Figure 2. Canine TCRG locus[86]

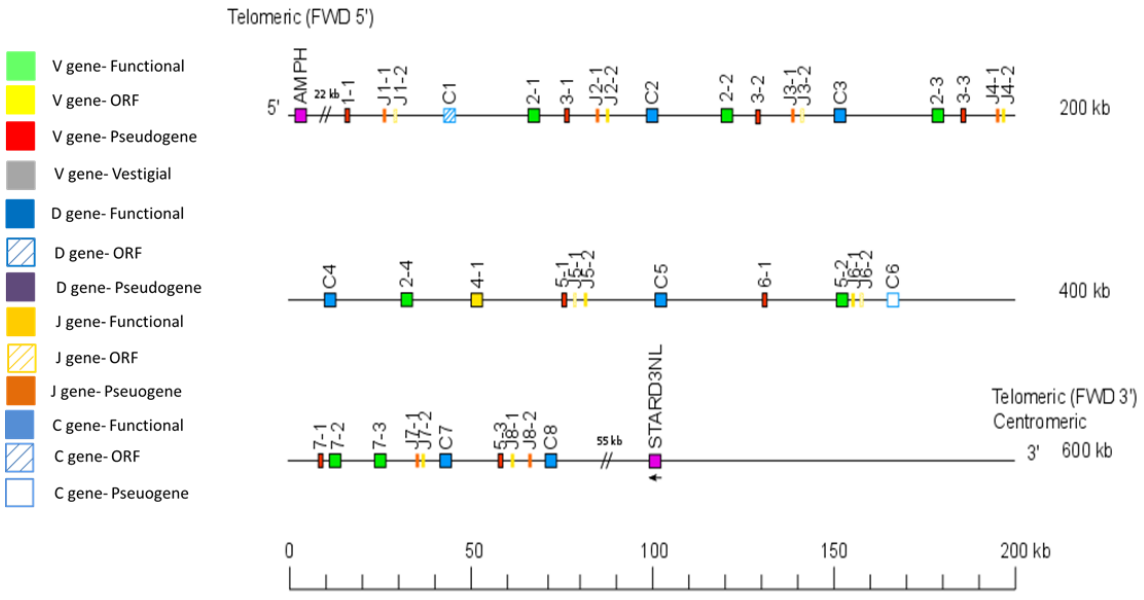
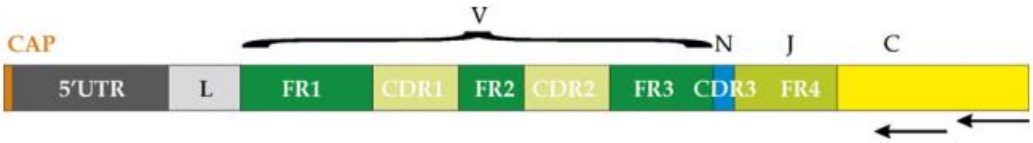


Figure 3. Schematic representation of the 5' end of Feline TRG cDNA as retrieved from Weiss et al. (2008)[75]. L, Leader sequence; FR, framework region; CDR, complementary determining region; V, variable region; N, n region; J, joining region; C, constant region.



Membrane Immunoglobulins (Ig) are antigen receptors expressed by B lymphocyte membranes, and the primary effectors of humoral immunity, which they can initiate without antigen presentation by other cells. These molecules are part of the antigen receptor complex in conjunction with Igα (CD79a) and Igβ (CD 79b). The structure comprises two Heavy chains (H) and two light chains (either κ or λ chain) encoded by IGH and IGK/IGL genes respectively. The heavy chain defines immunoglobulin class: in the case of membrane Ig, the presence of IgHμ or IgHδ determines either IgM or IgD subtype respectively[89]. Recognition and binding of antigens rely on the Fragment Antigen binding (Fab) region, composed of a variable (Fv) and a constant (Fb) segment of both heavy and light chains, similar to the TCR variable and constant domain respectively, and as well is encoded by genomic V and C regions. The V region is composed of four different conserved Framework Regions (FR1, FR2, FR3, FR4) intercut with three hypervariable complementary regions (CDR1, CDR2, CDR3)[90]. Immunoglobulin structure is illustrated in **Figure 4**.

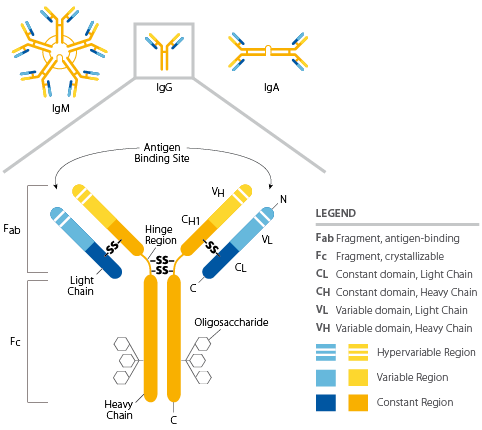


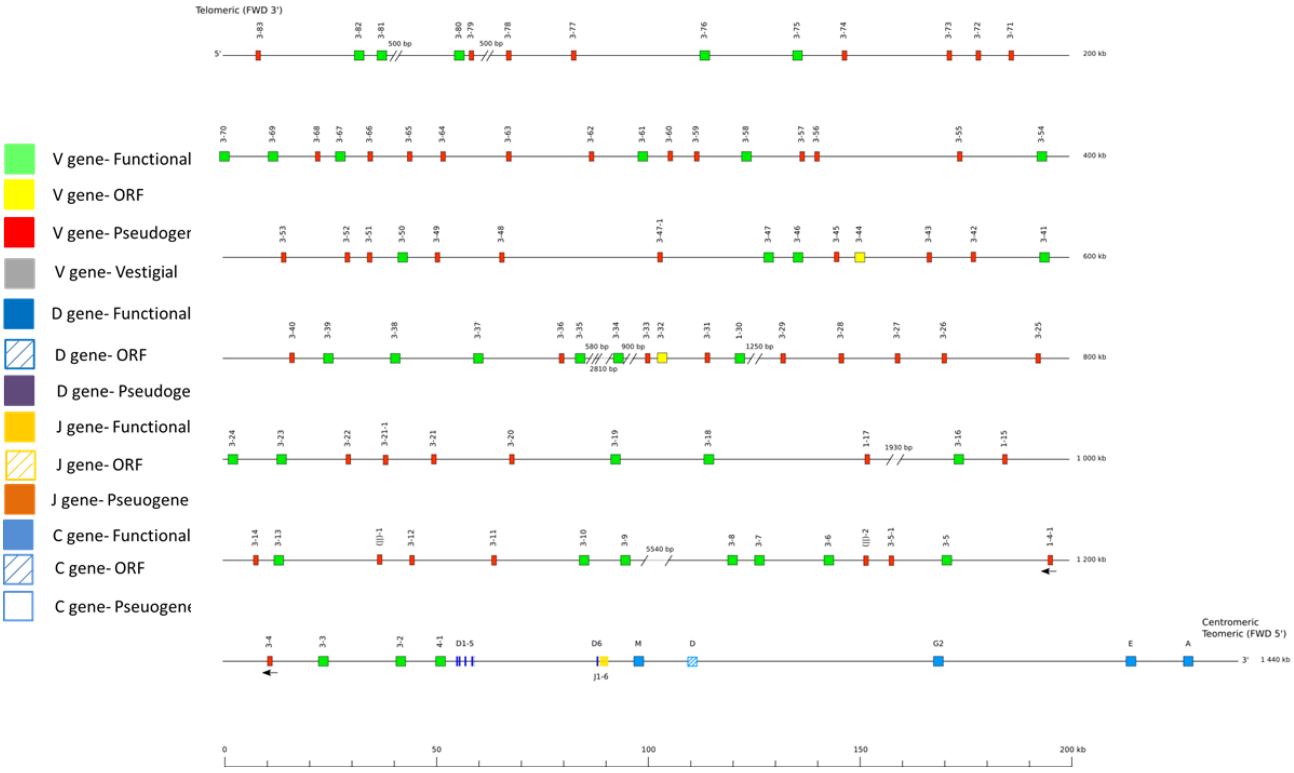
Figure 4. Immunoglobulin structure

The canine IGH gene has been extensively described. It is located on chromosome 8 and spans approximately 1400 kilobases (kb)[66]. The locus consists of four IGH-V genes families (for a total of 89 genes, of which 36 are functional), six IGH-D genes and six IGH-J genes organised in as many sets, and IGH.C genes. The locus is represented in **Figure 5**[86].

The full-length sequences of feline genes encoding for immunoglobulin heavy chains have not been thoroughly characterised. The V sequences have been so far ascribed to two subgroups, IGH1 and IGH3[91]. In humans, the highest number of V genes are part of

the IgH3 family followed by the IgH1[92]. This rule seems to be observed in the cat genome as well.[74]

Figure 5. The canine immunoglobulin heavy chain locus[86].



Knowledge on genetic structure is crucial in primer design. However, additional difficulties in PARR design other than the complexity of the AR genes, are the potential mutations occurring at primer binding sites. This is a frequent event especially in B cell lymphomas: B lymphocytes after antigen stimulation undergo additional mutations (somatic hypermutation).[58] In Human Medicine, it has been suggested as a possible cause of amplification lack in translocation during B cell lineage maturation, and to avoid this inconvenience it has been proposed to correlate standard primer sets directed to IGH genes, with supplementary primer sets directed to other targets (such as IGK).[63]

It is clear how an essential percentage of rearrangements is still likely not to be detected, due to the presence of unidentified VH and JH segments. The design of primers should be as broad as possible as it is crucial to avoid possible false-negative results.

False positive results can occur as well. If every neoplasm is clonal, not all clonal populations are neoplastic. It has been reported how some non-neoplastic diseases can yield a clonal population when analysed by molecular assays. Chronic infections such as canine Ehrlichiosis or Leishmaniasis or other vector-borne diseases[37], or other conditions[93] can stimulate a single or a few lymphocyte subclasses, giving a few clonal peaks that can lead to misdiagnosis of a reactive condition as neoplastic.

Moreover, the interpretation of results is crucial. Amplification products can be visualised by standard Agarose or PAGE gels, which represent dated techniques that nowadays are being replaced by more sensitive capillary electrophoresis (CE) detection methods. Although several attempts have been made in human medicine to standardise the interpretation of the peaks resulting after capillary electrophoresis[94–96], reporting clonality testing is still a challenge for the pathologist, and relies on experience and results from previous assays performed, especially in more challenging cases.

Despite these many limitations, PARR is a rich-of-potential technique. Research nowadays is focused on optimisation of the technique firstly, and on the application of PARR to other diagnostic purposes. For instance, detection of minimal residual Disease (MRD) and relapse of neoplasia in early stages is one of the promising applications for

the near future. Studies have been conducted on the dog, showing how PARR can be a useful tool in the detection of MRD[97,98] in dogs with remission of the disease with higher sensitivity than flow cytometry[99].

1.3. AIMS OF THE STUDY.

The primary aim of this study was to refine the diagnosis of lymphoma achieved by flow cytometry using PCR techniques (PARR).

This refined PCR method would then be compared to flow cytometry in the same set of subjects.

Both methods will be compared in dogs and cats with lymphoproliferative disease and non-malignant proliferative conditions.

These aims are based on the following:

1) The importance of immunophenotype in lymphoma/ leukaemia diagnostics,
2) The advantages regarding cost and time, flow cytometry could represent a valid alternative to other immunophenotyping techniques in the assessment of phenotype in lymphoproliferative diseases in both dogs and cats,
3) The sensitivity of PARR in detecting a clonal population within a reactive lesion,
4) The advantage of PARR which can be performable on virtually any sample type,
5) The lack of knowledge on feline clonality assays and the lack of comparison of this method with techniques other than IHC,
6) The study will be specifically aimed at refining the methods for PARR for use with a variety of tissue sample from cats and blood from dogs.

CHAPTER 2:

OPTIMIZATION OF THE DNA EXTRACTION TECHNIQUES.

2.1. INTRODUCTION.

Clonality assays are aimed to amplify specific genes expressed homogeneously by the cells examined, discriminating between the diagnosis of neoplasia or reactive lesion. Extraction and recovery of good quality genomic DNA (gDNA) is a critical juncture to obtain proper PCR amplifications and reliable results.

First known as "nuclein", DNA has been studied over several years during the second half of the twentieth century and was finally described by Watson and Crick in 1953[100,101].

Nevertheless, its first serendipitous extraction was performed about one century earlier by the German chemist Friedrich Miescher, who discovered this nucleus-derived molecule while studying cell chemical composition. The new substance was discovered in pus derived leukocytes, due to its insolubility in ether and its resistance to protease digestion. The first DNA extraction protocol was then set up over a few years and consisted of three crucial phases: isolation of cells of interest, isolation of nuclei and, finally, isolation of DNA. It was a long, time-consuming protocol, with the use of alkaline first, followed by acid solutions to enable precipitation of nucleic acids[102].

Nowadays, although different easy, time-saving commercial protocols have been developed, the necessary steps for DNA extraction are mostly invariant. Lysis is achieved using specific solutions aimed to reduce the disulfide bonds (such as dithiothreitol- DTT) or to mechanically dissolve the membrane, such as Sodium Dodecyl Sulfate (SDS) and heat to increase the fluidity of the cells and disrupt them. Chemical digestion by enzymes, such as Proteinase K, is also performed. After lysis is complete, DNA is bound to a solid phase, and the final elution step provides the pure gDNA solution.

Fundamental nucleic acids features when a molecular biology experiment is intended to be conducted, are: a good quantity of gDNA recovered expressed as ng/µL of eluate; purity of the gDNA recovered designed to have low contamination by proteins, salt and other substances; integrity of the gDNA recovered. Especially in a situation where archived (such as frozen blood, or cell pellets) or old (such as stained cytological or

haematological slides or FFPE tissue sections) samples are used, meeting these requirements can be challenging.

Different techniques are suitable for nucleic acid assessment. One of these is spectrophotometry, an analytical technique, which allows the quantification of a substance in a solution, depending on its own Ultra-Violet (UV) light absorbance spectrum[103,104]. The concentration of the molecule is calculated by the Modified Beer-Lambert Equation assuming that attenuation of a beam of light passing through it depends on the contents of solutes[104,105]. Concentration (c) of gDNA measured by spectrophotometry is described as:

$$c=(A*\varepsilon)/b$$

Where, A is the absorbance, intended as the measure of the attenuation of a beam of light passing through a solution; ε is the extinction coefficient, a measurement of how strongly a molecular species absorbs light at a given wavelength; b is the path length in cm, which is a peculiar characteristic for each spectrophotometer. For double-stranded DNA, absorbance maxima is 260nm (A_{260}), whereas the extinction coefficient is 50ng-cm/µl.[104]

Moreover, evaluation of the absorbance at different wavelengths allows assessment of contamination by other molecules such as proteins or salts. The purity of DNA is generally evaluated by considering absorbance at 280 nm (A_{280}), which is the absorbance maxima for proteins and at 230 nm (A_{230}), which is considered the absorbance maxima of phenol and guanidine, mostly found in commercial DNA extraction kits and often residual from the extraction process. Ratios of A_{280} and A_{230} with A_{260} represent contamination by protein (given by $A_{260/280}$ ratio) and phenol/ guanidine (given by $A_{260/230}$ ratio), respectively.

Pure gDNA has an A_{260}/A_{280} around 1.8 and an A_{260}/A_{230} of around 2. Lower values are indicative of contamination or residual reagents from the extraction process. Higher values are usually consistent with measuring on a contaminated instrument, or RNA

contamination[105]. It's important to note that extremely low concentrations can lead to incorrect ratio measurements[105].

The last important characteristic to consider when approaching DNA analysis is gDNA integrity. Polymerase Chain Reaction (PCR) amplification of control genes distributed across the entire genome can serve this aim[106]. Other techniques include quantitative PCR amplification such as Real Time PCR[107–109], nested-PCR[110], or simple, direct visualisation of DNA on agarose gels[109].

This part of the study has been designed to optimise DNA extraction protocols to use for further experiments. We selected the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich)[111] and the QIAamp DNA Blood Mini Kit (Qiagen)[112]. We aimed to find the best protocol using the minimum amount of sample possible and obtaining the maximum nucleic acid yield. We focused our attention on EDTA whole blood and cytological slides as source material, using the archive of the Central Diagnostic Services (University of Cambridge).

2.2.MATERIALS AND METHODS.

2.2.1. EDTA BLOOD SAMPLES.

Two different kits provided the protocols selected for the trial: the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich)[111] and the QIAamp DNA Blood Mini Kit (Qiagen)[112].

In a preliminary phase, archived EDTA blood samples were processed according to the manufacturer's instructions. Afterwards, each sample was treated by four optimised protocols (illustrated below), aimed to increase DNA yield with minimum contamination from reduced sample volumes.

We investigated the following: 1) the best performance for the same volume of sample used (200µl as recommended by the manufacturer) and which method had the best overall output between the two kits; 2) if the number of cells used affected DNA

retrieval and if lowering cell counts suitable amounts of gDNA were still achievable; 3) which method assured the lowest levels of residual contamination by reagents.

2.2.1.1 Case selection.

Twenty-two EDTA blood samples were retrieved from the Central Diagnostic Services (University of Cambridge). All the samples were stored at -80° C for a maximum of one year.

Ten samples both from dogs (five) and cats (five) with a mild increase in WBC were enrolled in the first part of the trial: the aim was to compare the yield of the two kits using the manufacturer's protocol (**Table 1**).

The other twelve canine blood samples showed an increase in WBC, and were further subcategorised into three groups to facilitate the following statistical analysis: (1) a mild increase in WBC (count of 6-50x10^9/mL), (2) a moderate increase (count of 50-100 x 10^9/mL) and (3) marked increase (count >100 x 10^9/mL). These cases were all previously diagnosed with hematopoietic neoplasia: B cell chronic lymphoid leukaemia (B-CLL) and undifferentiated acute leukaemias (AUL) in six cases (three per subtype of leukaemia); Large Granular Lymphocyte Chronic Leukaemia (LGL-CLL) and T cell CLL (T-CLL) in four cases (two per each subtype of leukaemia), T cell Acute Leukaemia (T-ALL,) and Acute Myeloid Leukaemia (AML) in the remaining three cases (**Table 2**).

As we intended to use the Qiagen kit we preferred to optimise this protocol. In fact, these twelve samples were processed using four different protocols: first, the Qiagen protocol with no modifications (protocol QA); secondly, the Qiagen protocol using volumes of blood containing 5x10^6/L of WBC (protocol QB); thirdly, the Qiagen protocol using amounts of blood containing 9x10^6/L of WBC (protocol QC); finally, the Sigma kit, using volumes of blood containing 9x10^6/L of WBC (protocol S).

2.2.1.2. Protocols.

2.2.1.2.1 GenElute Mammalian Genomic DNA Miniprep Kit (Protocol Sigma).

A mixture of 20µL of the Proteinase K (Sigma Aldrich) and 200µL of EDTA blood was placed into a 1.5mL microcentrifuge tube. To obtain RNA-free samples incubation for 2 minutes at room temperature (RT) with 4µL of RNase A Solution (Qiagen) was carried out. Lysis was performed by adding 200µL Lysis Solution C (provided in the kit) to the mixture and incubating at 55 °C for 10 minutes. Each pre-assembled GenElute Miniprep Binding Column was prepared with 500µL of the Column Preparation Solution which was discarded after centrifugation at 12.000 ×*g* for 1 minute. This step is considered essential for maximisation of gDNA binding to the column. Two-hundred µL of pure (96/100%) ethanol was added to the sample, which was consequently spun at ≥6500 × *g* for 1 minute. After discarding the flow through, two washing steps were performed, using the Washing Buffer provided in the kit. The final elution was carried out adding 200µL of the Elution Solution (provided) directly into the centre of the binding column; before centrifugation, the elution buffer was incubated in the column for five minutes at room temperature, to increase the elution efficiency.

2.2.1.2.2 QIAMP Mini and Blood kit (Protocol QA).

Digestion of 200µL of whole EDTA blood was carried out using 20µl of proteinase K. To obtain RNA-free samples incubation for 2 minutes at room temperature (RT) with 4µL of RNase A Solution (Qiagen) was carried out. Lysis was performed by adding 200µL of AL buffer (provided), incubation for 10 minutes at 56°C followed by the addition of 200µL pure (96-100%) of ethanol to the sample. No preparation was needed for the column in this kit. The lysate was transferred to the QIAprep Spin Miniprep Columns (Qiagen), and after centrifugation, the flow through was discarded. Washing steps were then performed twice with two different washing buffers (buffer AW1 and AW2 both provided). Elution was carried out using 200µL of elution buffer. As recommended by the manufacturer, a second elution was also performed in the same column, with a total final volume of eluate of 400µl.

2.2.1.2.3.Optimized protocols (protocols QB, QC, S).

The optimisation of the protocols was aimed to use the minimum amount of sample possible. We chose to extract gDNA using both kits, from volumes corresponding to WBC of $9x10^6$/L (protocols QC and S) and $5x9x10^6$/L (protocol QB). In order to have the 200µL volume recommended by the original protocols, volumes were adjusted to the cell count: if the desired cell count was contained in less than 200µL, the final volume was made up using PBS. If more than 200µl of blood was required, 200µL of PBS was added after obtaining a cell pellet by centrifugation.

Moreover, a smaller amount of elution buffer was used (150µl) to increase the final concentration of gDNA.

Table 1. Group 1. Patients affected by mild WBC increases. Blood was processed according to GenElute Mammalian Genomic DNA Miniprep Kit (**protocol Sigma**) and QIAamp DNA Blood Mini Kit (**protocol QA**) protocols.

Sample ID	WBC (x10^6/L)	Species	Protocol used	Volume (µL)
1Q 1S	6.16	Dog	QA Sigma	200
2Q 2S	21.47	Dog	QA Sigma	200
3Q 3S	8.63	Dog	QA Sigma	200
4Q 4S	9.36	Dog	QA Sigma	200
5Q 5S	10.08	Dog	QA Sigma	200
6Q 6S	11.03	Cat	QA Sigma	200
7Q 7S	7.62	Cat	QA Sigma	200
8Q 8S	8.45	Cat	QA Sigma	200
9Q 9S	18.14	Cat	QA Sigma	200
10Q 10S	39.14	Cat	QA Sigma	200

Table 2. Second group of patients, affected by leukaemia. Each sample was submitted to four different protocols. **QA**, Qiagen protocol according to manufacturer's instruction; **QB**, Qiagen protocol using volumes corresponding to a WBC count of $5x10^6$/L; **QC**, Qiagen protocol using volumes corresponding to a WBC count of $9x10^6$/L; **S**, Sigma protocol using volumes corresponding to a WBC count of $9x10^6$/L. Volume of blood and PBS were adjusted to have the final WBC and the final volume of 200µL.

Sample ID	WBC ($x10^6$/L)	Species	Diagnosis	Protocol Used	Volume (µl)	PBS (µl)
11QA	256.2	Dog	B-CLL	QA	200	
11QB	5			QB	19.5	180.5
11QC	9			QC	35.1	164.9
11S	9			S	35.1	164.9
12QA	32.9	Dog	LGL-CLL	QA	200	
12QB	5			QB	152	48
12QC	9			QC	273	
12S	9			S	273	
13QA	203.99	Dog	T-CLL	QA	200	
13QB	5			QB	24.5	175.5
13QC	9			QC	44.1	155.9
13S	9			S	44.1	155.9
14QA	52.56	Dog	LGL-CLL	QA	200	
14QB	5			QB	95.1	104.9
14QC	9			QC	171.2	28.8
14S	9			S	171.2	28.8
15QA	121.43	Dog	AUL	QA	200	
15QB	5			QB	41.2	158.8
15QC	9			QC	74.1	125.9
15 S	9			S	74.1	125.9

Table 2. Continued.

Sample ID	WBC (x10^6/L)	Species	Diagnosis	Protocol used	Volume (µL)	PBS (µL)
16QA	258.36	Dog	AUL	QA	200	
16QB	5			QB	19.4	180.6
16QC	9			QC	34.8	165.2
16 S	9			S	34.8	165.2
17QA	90.9	Dog	AML	QA	200	
17QB	5			QB	55	145
17QC	9			QC	99	101
17S	9			S	99	101
18QA	27.69	Dog	T-ALL	QA	200	19.4
18QB	5			QB	180.6	
18QC	9			QC	325	
18S	9			S	325	
19 QA	77.23	Dog	B-CLL	QA	200	
19QB	5			QB	64.7	64.7
19 QC	9			QC	116.5	83.5
19 S	9			S	116.5	83.5
20 QB	76.96	Dog	AUL	QA	200	
20 QB	5			QB	65	135
20 QC	9			QC	83.1	116.9
20S	9			S	83.1	116.9
21 QA	19.09	Dog	T-CLL	QA	200	
21 QB	5			QB	471.5	
21 QC	9			QC	261.9	
21 S	9			S	261.9	
22 QA	38.1	Dog	B-CLL	QA	200	
22 QB	5			QB	128.5	71.5
22QC	9			QC	231.3	
22 S	9			S	231.3	

2.2.2. CYTOLOGICAL SLIDES.

The kit used for the trial was the QIAamp DNA Blood Mini Kit (Qiagen)[112].
Archived cytological slides from dogs diagnosed with lymphoma/leukaemia were processed using the manufacturer's instruction and two protocols published by Roy-Chowdhury et al. (2016)[113] and Killian et al. (2010)[114].
In particular, the aim of the experiment was: 1) to evaluate how many slides per patient are required to obtain the best gDNA yield and purity; 2) to indicate how to select slides according to characteristics: in particular, the amount of material assessed macroscopically and the cellularity needed to have the best gDNA yield and purity; 3) to determine which method assured the lowest levels of residual contamination by reagents; 4) to combine all the information to choose the best protocol.

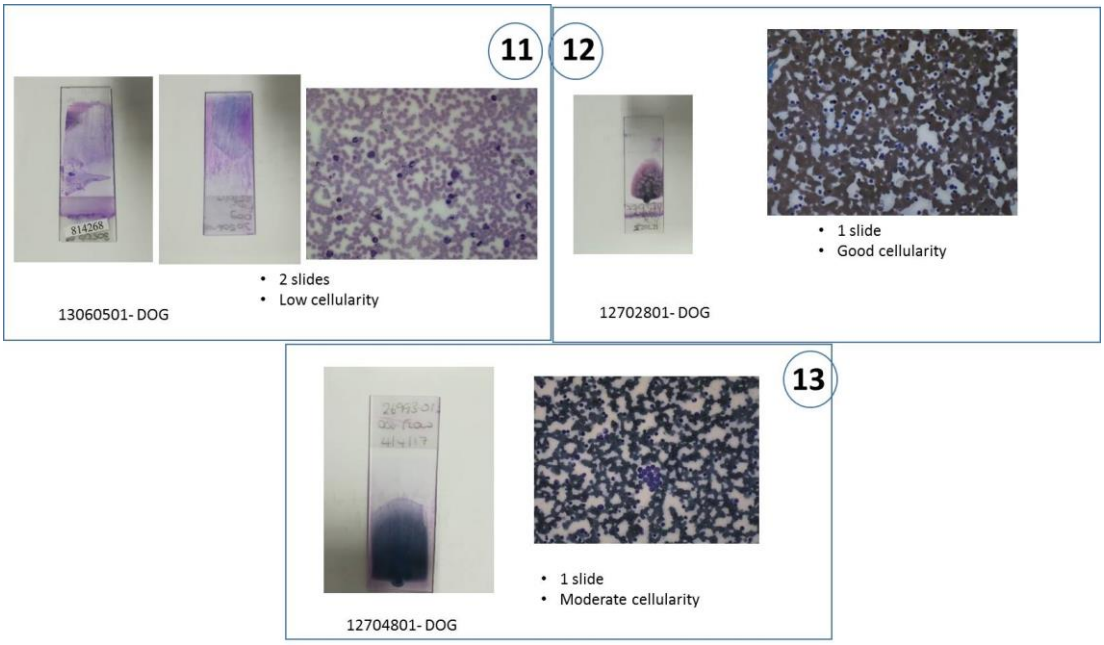
2.2.2.1. Case selection.

Blood smears slides from 12 cases were selected for inclusion in the study based on subjective microscopic determination of cellularity of the smears and the morphological condition of the cells (classified as high, moderate and low cellularity). Two slides were available for seven patients (cases no. 3, 4, 6, 7, 8, 1, 14), whereas a single slide was available for the rest of the cases (no. 5, 9, 10, 12, 13). The macroscopic amount of material was moderate in the majority of the cases (no. 3, 4, 8, 9, 12, 13) while the remainder had low cellularity. Finally, cellularity was high in two cases (no. 7, 12), moderate in five (no. 3, 4, 5, 8, 13) and low in two slides (cases no. 9, 11). An example of cellularity of the slides is shown in **Figure 6**. The slides were assigned to three different groups, submitted to the three protocols: group one (samples no. 3, 4, 5, 6) using the manufacturer's instructions; group two (samples no. 7, 8, 9, 10) using the protocol from Roy-Chowdhury et al.; and group three using Killian et al. protocol (samples no. 11, 12, 13, 14). Case data are summarised in **Table 3**.

Table 3. Cytological slides used to optimise the DNA extraction protocol.

Sample ID	N. of slides	Species	Cellularity	Protocol used	Elution step	Final elution volume (µL)
3	2	Dog	Moderate	Qiagen[112]	Single	100
4	2	Dog	Moderate		Single	100
5	1	Dog	Moderate		Single	100
6	2	Cat	Moderate		Single	100
7	2	Dog	Good	Roy- Chowdhury et al.[113]	Double	100
8	2	Dog	Moderate		Double	100
9	1	Dog	Low		Double	100
10	2	Cat	Moderate		Double	100
11	2	Dog	Low	Killian et. al.[114]	Single	100
12	1	Dog	Good		Single	100
13	1	Dog	Moderate		Single	100
14	2	Cat	Moderate		Single	100

Figure 6. Examples of cellularity and macroscopic appearance of the slides: sample no. 11, two slides, low cellularity; sample no. 12, one slide, good cellularity; sample no. 13, one slide, moderate cellularity. All these samples were from canine patients and were submitted to the Killian et al.[114] extraction protocol.



2.2.2.2. Protocols.

2.2.2.2.1. Manufacturer's instructions (QIAamp DNA Blood Mini Kit, Qiagen).

The dried material of each slide was moistened using a drop (~20µL) of PBS and carefully scraped off using a clean slide into a microcentrifuge tube containing 180µL of PBS. Digestion with 20µL of proteinase K (Qiagen Valencia, CA) and 4µL of RNAse (incubated for 2 minutes at RT), was carried out. Lysis was performed by adding 200µL of Buffer AL (provided in the kit) and incubated at 56°C for 10 minutes. Then, 200µl of pure (96-100%) ethanol was added and the mixture placed into the QIAmp mini spin column. First centrifugation was performed at 6000xg (8000 rpm) for 1 minute. After discarding the flow through the liquid, two consequent washing steps using Buffer AW1 and AW2 (both provided in the kit) were performed. Finally, buffer AE was added to the column and incubate at RT for five minutes. The final centrifugation allowed 100µL of eluate containing the gDNA to be obtained.

2.2.2.2.2. Protocol from Roy-Chowdhury et al. 2016.[113]

The material on the slide (s) was scraped off without preliminary moisturising into a 1.5mL microcentrifuge tube. Lysis was performed using 180µL of buffer ATL (provided), and 20 µL of Proteinase K (Qiagen Valencia, CA), with overnight incubation at 56° C. Purification was performed according to the kit protocol, and a double elution with 50 µL of elution buffer (AE, provided) was performed.

2.2.2.2.3. Protocol from Killian et al. 2010.[114]

A small amount (20µL) of Buffer ATL (provided) was used to help the consequent scraping off the slide of the material. The material was then placed in a 2mL collection tube, where lysis was performed by adding a 100µL solution of a 4:1 mixture of ATL buffer (provided) and Proteinase K (Qiagen Valencia, CA) and incubating at 60°C for 2 to 16 hours (an average of 7 hours). RNA was then degraded by incubation with 4µL of RNase solution, and column purification was performed. Final elution was made using 100µL of Buffer AE (provided).

2.2.3. DNA QUALITY ASSESSMENT.

Assessment of quantity (expressed by gDNA concentration) and purity was performed by spectrophotometry, while detection of control genes was performed by PCR amplification.

2.2.3.1. Spectrophotometry.

Spectrophotometry is considered a reliable technique to assess concentration and quality of the DNA contained in the eluate [115]. A Nanodrop 1000 (ThermoFisher Scientific) was used in the present study.

A total of 2µl of DNA solution was pipetted directly onto the measurement pedestal. The sampling arm was then lowered, and the sample was maintained in place between the two optical fibres by the surface tension. The corresponding elution solution used for the specific sample was loaded and measured as a blank before each analysis.

DNA concentration was expressed as ng/µL. Concentration was defined as low (<20 ng/µL), intermediate (20-30ng/µL), good (> 30 ng/µL).
To assess protein contamination, the ratio ($A_{260/280}$) between absorbance at 260 mm wavelength (considered the maximum for nucleic acids[104]) and 280 (considered the maximum for protein) was determined. Contamination by phenol and salts was assessed using the ratio value for 260 and 230 mm wavelength absorbance ($A_{260/230}$). A value of $A_{260/280}$ around 1.8 and a value of $A_{260/230}$ around 2.0 are indicative of pure DNA[105]. We considered the sample of good overall quality if the concentration was >30 ng/µL, $(A_{260}/A_{280}) \geq 1.8$ and $(A_{260}/A_{230}) \geq 2$.

2.2.3.2. Polymerase Chain Reaction (PCR).

To check the suitability of the DNA obtained to undergo further analysis PCR of known genes was performed both for canine and feline patients. The reaction was conducted on a thermocycler, which cyclically increases and decreases the temperature of the samples, to allow the denaturation of the DNA, annealing with the primer and amplification. The reaction is enabled by the use of optimised concentrations of MgCl, DeoxynucleotideTriphosphate (dNTPs), a PCR buffer, and a DNA polymerase (HotStarTaq Plus, extracted from the bacterium *Thermus aquaticus*[116], stable at high temperature).

Control PCR on canine samples was performed using a primer directed to the juxtamembrane region of the canine C-kit gene, with the following sequence[67]:

forward primer: 5' CCC ATG TAT GAA GTA CAG TGG AAG 3'

reverse primer: 5' GTT CCC TAA AGT CAT TGT TAC ACG 3'.

PCR amplification was carried out using the Type-it ® Mutation Detect PCR kit, according to the manufacturer's instructions[117]. The protocol is briefly described below. A mixture of 12.5µL Type-it Multiplex PCR Master Mix, 2.5µL of Q solution, 2.5µL of Coral Load Dye 10x, and variable amounts of eluate containing template gDNA and RNAse-free water is loaded in individual PCR tubes, one for each sample to be analysed. The volume of eluate added has to contain an amount of nucleic acid around 100-120 ng/µl, and the volume of water is calculated to reach a total amount of 25µl. After the initial activation of the polymerase at 95°C for 5 minutes, 35 same cycles of denaturation (95°C for 30 seconds), annealing (64°C for 60 seconds) and extension (72°C for one minute) are performed. A final step of extension is carried out at 68°C for 10 minutes.

The feline Androgen Receptor (fAR) gene was used for normalisation of feline DNA, as suggested by Mochizuki et al.[77]and was performed using the following sequence:

forward primer:5' CAC AAT GCC GCT TAC GGG GAC CT 3';
reverse primer: 5' AGG GGG TCA CAG ACC CTG ACT CG 3'.

PCR amplification was carried out using the TopTaq ® Master Mix kit, according to the manufacturer's instructions[118]. The protocol is briefly described below. A mixture of 12.5µl TopTaq multiplex PCR Master Mix, 2.5µL of Coral Load Dye 10x, one µl of 50x primer mix was prepared and loaded into PCR tubes, one per each reaction. A variable amount of eluate containing template DNA and RNase-free water was then added assuring an amount of nucleic acid around 100-120 ng/µL and a total reaction volume of 25µL.

Amplification cycles included: 1) a first polymerase activation step (95°C for 5 minutes); 2) 40 cycles of denaturation (30 seconds at 95°C), annealing (90 seconds at 68°C) and extension (30 seconds at 72°C). A final extension at 68° C for 10 minutes.

Negative control reactions adding RNase-free water were ran in order to exclude false positive results due to contaminations of reagents.

Agarose gel electrophoresis was performed to visualise the PCR products. GelStar Stain (Lonza) was added to the 2 % agarose-TAE solution during gel casting, in the concentration of 1x. For quantification of the amplicons, DNA rulers were loaded to the external wells of the gel: FastRuler Ultra Low Range DNA Ladder and FastRuler Low Range DNA Ladder (Thermo Fisher Scientific). A total of 5µL of sample was loaded in each well, and the samples were run at a voltage of 80V for 60 minutes. Gels were scanned using the Gel Doc™ XR+(BioRad) scanner, and the excitation and emission were set at 493 nm and 527 nm respectively, according to the stain requirements[119].

Feline DNA was considered suitable for further analysis if the reaction yielded a single distinct band of around 189 bp [77]. For dogs, the expected band size after amplification was approximately 200 bp[67].

2.2.4. STATISTICAL ANALYSIS.

Descriptive statistics were used to analyse the results. The continuous variables considered were: concentration and WBC. To correlate categorical variables a chi-square test was used; a Student T-test to correlate one level categorical and continuous variables; One-way ANOVA test was used if the categorical variable had more than two levels, with a Tukey test as Post-Hoc test. Finally, Pearson's test was used to correlate continuous variables. Significance was set with a p-value < 0.05.

2.3. RESULTS.

2.3.1. Extraction of DNA from EDTA blood.

Eluates from three samples (sample no. 15, 20, 21) were excluded from further analysis, due to high blood contamination, macroscopically evaluable. For the rest of the samples were statistically analysed.

The median concentration of gDNA obtained was 33.13ng/µL, with a maximum concentration of 137.46ng/µL and a minimum of 5.76ng/µL. No significant difference was evident between GenElute Mammalian Genomic DNA Miniprep Kit Protocol (Sigma Aldrich) and the QIAamp DNA Blood Mini Kit (Qiagen) protocols, and no significant difference was found if an optimised method was used. On the other hand, a variable that has a direct impact on the final DNA concentration is the initial cell count of the sample: using 200 µL of whole blood, it is more likely that lower counts give eluates with a lower DNA concentration ($p=0.001$); if the volume is adjusted in order to have $9x10^6$ or $5x\ 10^6$ cells/L, there is no difference in final DNA concentration, allowing the reduction of sample volume used. Moreover, samples with a WBC concentration of $9\ x10^6$ gave significantly ($p= 0.047$) better overall quality gDNA, when processed using the GenElute™ Mammalian Genomic DNA Miniprep Kit Protocol (Sigma) (**Figure 8**). All the protocols gave good results regarding quality (protein, phenol and salt

contamination) and all the samples were positive to the amplification reaction of control genes, canine C-kit and fAR. (**Figure 7**).

Data from the Extraction protocols are illustrated in **Table 4**.

Figure 7. The positive reactions of samples no. 11, 12, 13, 14 canine C-kit gene (**A**) and fAR (**B)** gene. Single prominent bands are evident in the expected area of the gel. (2% Agarose gel, GelStar™ Stain, 80 V, 60 mins).

Figure 7A.

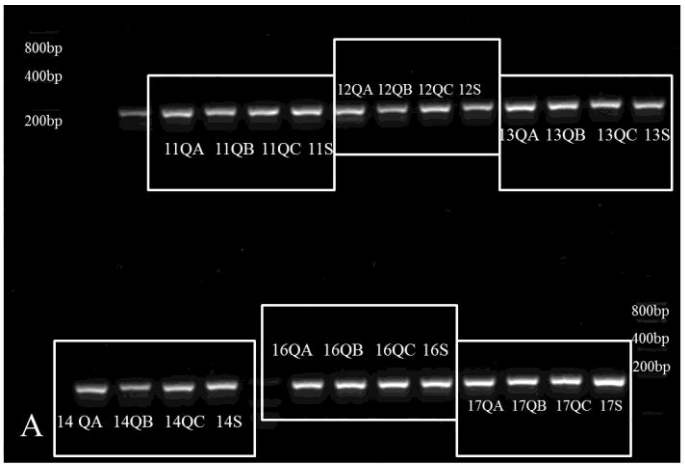


Figure 7B.

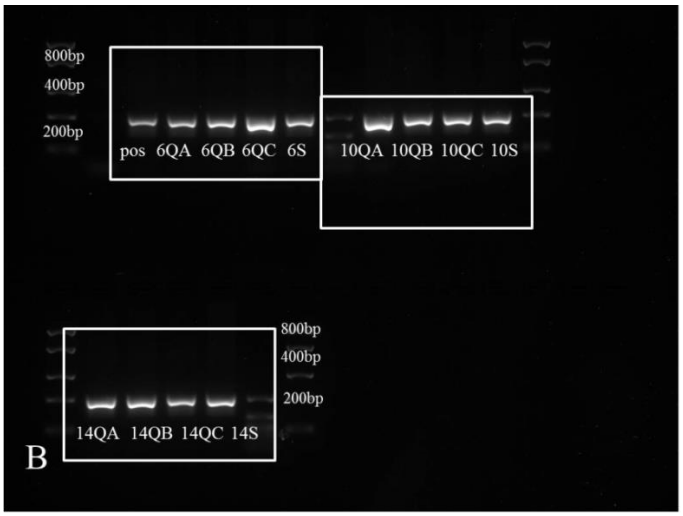


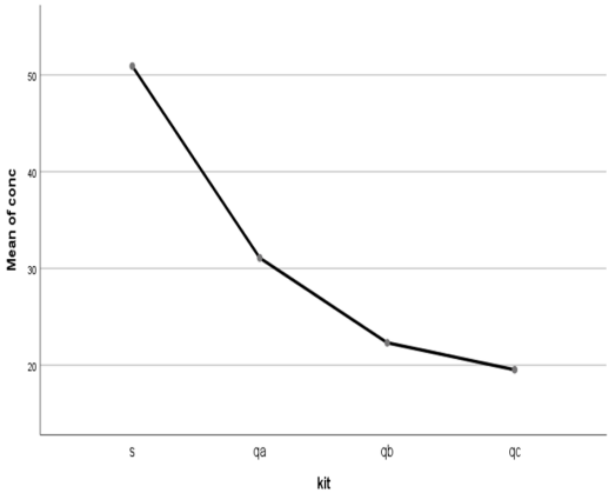
Table 4. Extraction data of EDTA blood samples using two kits, S (Sigma) and Q (Qiagen with three protocols: A,B,C).

Sample ID	kit used	DNA concentration (ng/µL)	$A_{260/280}$ ratio	$A_{260/230}$ ratio
1	QA	6.5	2.585	2
	S	12.265	2.225	1.83
2	QA	35.6	1.98	2.13
	S	26.8	1.82	2.11
3	QA	13.4	1.77	1.43
	S	18.75	1.945	1.04
4	QA	6.8	1.35	1.54
	S	16.8	1.9	1.805
5	QA	18.7	1.675	1.96
	S	16.1	1.535	1.51
6	QA	19.45	1.76	1.4
	S	26.5	1.66	1.5
7	QA	9.5	152	1.5
	S	15	1.71	1
8	QA	10.7	1.77	4.43
	S	17.2	1.94	1
9	QA	12.85	1.47	1.6
	S	32.3	1.74	1.3
10	QA	35.1	1.7	1.8
	S	23	1.6	1.3
11	QA	22.16	1.695	1.045
	QB	13.14	1.875	1.565
	QC	17.77	1.73	1.985
	S	59.41	1.85	2.12
12	QA	26.85	1.97	2.16
	QB	29.61	1.875	1.555
	QC	13.24	1.77	1.185
	S	71.86	1.63	1.77
13	QA	52.13	1.925	1.635
	QB	53.89	1.87	1.975
	QC	35.43	1.635	0.47
	S	66.97	1.775	1.17
14	QA	13.78	1.857	1.525
	QB	10.75	1.625	1.425
	QC	7.31	2.365	1.38
	S	33.64	1.715	0.96
16	QA	137.46	1.895	1.93
	QB	20.66	1.83	2.695
	QC	33.18	1.82	3.395
	S	120.21	1.83	2.165

Table 4 (Continued). Extraction data of EDTA blood samples using two kits, S (Sigma) and Q (Qiagen with three protocols: A,B,C).

sample ID	Kit used	DNA concentration (ng/µL)	$A_{260/280}$ ratio	$A_{260/230}$ ratio
17	QA	22.55	1.89	2.25
	QB	15.85	2.01	1.64
	QC	27.18	1.785	1.985
	S	52.29	1.795	1.52
18	QA	33.93	1.805	2.135
	QB	34.77	1.785	2.145
	QC	16.54	1.62	2.035
	S	51.06	1.725	1.875
19 QA	QA	30.45	1.975	3.745
	QB	12.61	1.88	5.02
	QC	19.2	2.05	1.87
	S	103.6	1.775	1.775
22	QA	14.8	2.04	1.42
	QB	10.21	2.09	2.1
	QC	5.76	1.83	4.36
	S	62.9	1.85	1.865

Figure 8. Comparison of means of concentration obtained using different protocols. Protocol S yielded significantly higher DNA concentrations than protocol QC. Remarkably, the two protocols used the same volumes of EDTA blood with the same WBC ($9x10^6$/mL). This protocol, however, uses lower amounts of elution buffer, possibly allowing better binding of DNA and thus higher yield.



kit	kit	Mean Difference	Std. Error	Sig.
S	QA	19.826	10.280	.232
	QB	28.599	11.620	.081
	QC	31.390*	11.620	.047
QA	S	-19.826	10.280	.232
	QB	8.773	11.620	.874
	QC	11.564	11.620	.753
QB	S	-28.599	11.620	.081
	QA	-8.773	11.620	.874
	QC	2.791	12.821	.996
QC	S	-31.390*	11.620	.047
	QA	-11.564	11.620	.753
	QB	-2.791	12.821	.996

2.3.2. Extraction from cytological slides.

The median concentration of gDNA obtained was 14.4 ng/µL with a minimum of 1.7 ng/µL (obtained with the Qiagen protocol) and a maximum of 46.94 ng/µL (with the second protocol by Roy-Chowdhury et al[113]). DNA yields were in general very low, possibly due to inexperience with the technique. All the other results could be impaired by the low DNA concentrations: measurements of $A_{260/280}$ and $A_{260/280}$ can be impaired by low concentrations. No differences were found in yields and quality between kits used for DNA extraction. The only variable which seems to affect the final results was the elution method: the elution step. In fact, in the protocol provided by Qiagen and in the protocol supplied by Killian et al.[114], the elution was performed using 100µL in a single step. Roy-Chowdhury et al. instead used two elution steps, adding 50 µL each time, for a final total volume of 100µL. No other variable influenced the final DNA amount extracted.

Within samples extracted with the Qiagen protocol, only sample 5 and 6 showed a single band respectively identifiable with canine C-kit gene and fAR gene. Samples 8, 9 and 10, extracted with Roy-Chowdury's protocols were monoclonal for C-kit. Only sample 14 was strongly clonal within the third group (**Figure 9**).
Extraction data are shown in **Table 5**.

Table 5. Extraction data of cytological slides submitted to the three DNA extraction protocols.

Sample ID	Protocol used	Concentration (ng/µL)	$A_{260/280}$ ratio	$A_{260/230}$ ratio
3	Qiagen	14	1.15	2.22
4		4	1.64	2.93
5		1.6	1.2	2.35
6		11.12	1.92	2.2
7	Roy- Chowdury et al.	20.91	1.13	2.21
8		8.38	1.4	2.11
9		3.32	1.49	2.045
10		49.23	1.45	1.4
11	Killian et. Al	2.22	1.3	1.94
12		13.88		2.04
13		8.23	1.86	3.15
14		41.15	1.61	2.055

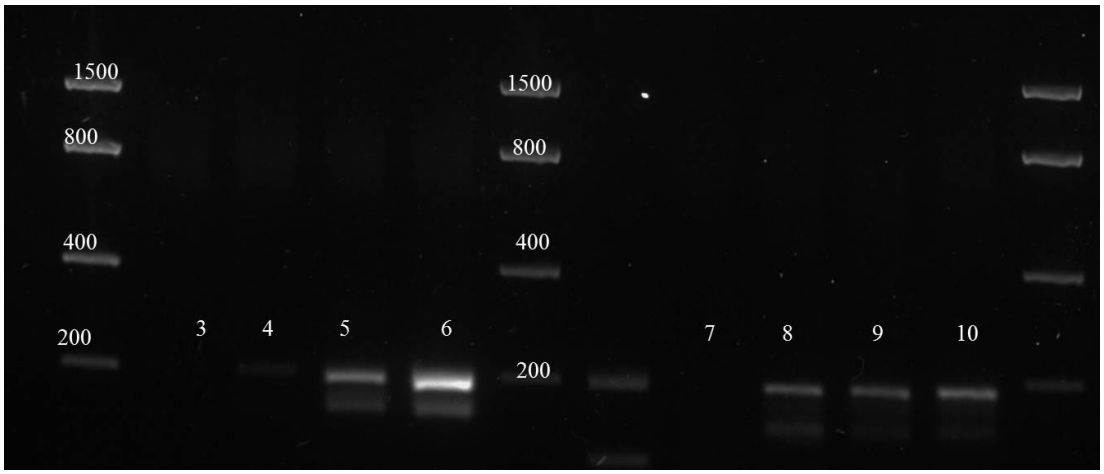
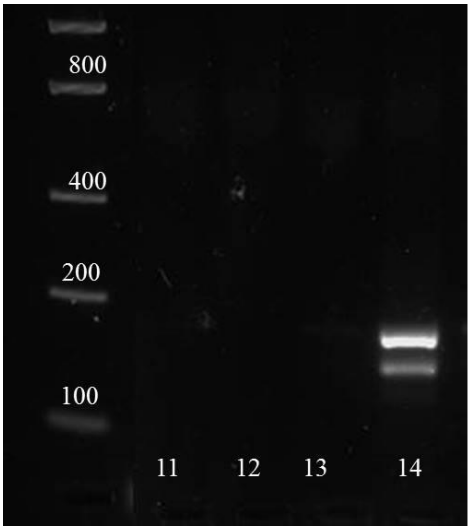


Figure 9. Visualisation of PCR products from amplification of canine C-kit gene and AR**.** NTC. Size rulers are run along with the samples in all the gels.



-***Qiagen Protocol:*** **Sample 3 and 4**, (C-kit), negative; **Sample 5**, (C-kit), positive; **Sample 6**, (fAR), positive.

-***Roy-Chowdhury Protocol***: **Sample 7**, (C-kit), negative. **Samples 8, 9**, (C-kit), positive. **Sample 10**, (fAR), positive.

- ***Killian Protocol***: **Sample 11, 12, 13**, (C-kit), negative; **Sample 12**, (fAR), positive.

2.4. DISCUSSION.

In this chapter, different DNA extraction techniques were investigated to choose the most suitable for our purposes.

Our results were encouraging when DNA was extracted from whole blood, showing how this kind of specimen is preferred to cytological slides. Fresh samples give more satisfying results, as cells and nucleic acids are less likely to be damaged or degraded. Cytological slides are reported to be a good source of gDNA[113,114,120,121], but the preservation is a crucial variable which can affect the final results. In our trial, the slides were old stained samples, stored with no special precautions. Possibly extraction from newer slides would have led to better results.

Moreover, we believe that Elution Solution volumes can be the keystone for a better extraction method. Lower elution buffer volumes than the recommended, are preferred in most of the protocols investigated[113,114]. Possibly, lower volumes allow a more efficient DNA binding and transfer into the solution. In our experience, therefore, a double elution step could provide better yields: most of the protocols recommend the second elution, and we think that eluting the columns twice with the same eluate, could improve the results.

Cellularity of the source material is of course decisive. Whole blood samples are highly cellular, and not comparable to a cytology slide. Even lower blood WBC volumes gave better results, showing that high cell counts are inevitably connected with higher gDNA levels. Although cytology slides are of lower cellularity than blood samples, they are usually available retrospectively from laboratory slide archives. They can be retrieved for DNA recovery if PARR analysis is indicated to aid or clarify a diagnosis made by cytology, ICC or FC and fresh sample is no longer available. This was the basis for this retrospective study of DNA extraction from archived cytology sides.

However, we believe in the potential of cytological slides as source material for molecular analysis: in our case, we consider PCR for antigen receptor rearrangement (PARR) a complementary tool in diagnostics of lymphoma/ leukaemia, enriching the immunophenotypic diagnosis. Molecular diagnostics from slides could solve many problems regarding availability and shipment of the samples, which can be processed

even if not fresh and which can provide suitable material if the original sample has been entirely used for other analyses (e.g. CBC, flow cytometry, etc.)

2.5. CONCLUSIONS.

We adopted the GenElute Mammalian Genomic DNA Miniprep Kit Protocol (Sigma) to extract DNA from EDTA whole blood, given the higher yield and the quality level assured by this extraction method. Moreover, we preferred to use volumes containing $9x10^6$/mL as the kit gave significantly better results at this concentration. Final protocols will be illustrated in Chapter 4 of the thesis.

Nucleic acid extraction from slides was not as successful as expected during this trial. However, we felt confident to reduce the elution volumes to 50µL and to perform the double elution of the columns, based on the protocol from Roy-Chowdury et al[113]. Moreover, we decided to perform the lysis according to Killian et al.[114] protocol, which performs first lysis of the material directly on the slide, using the tissue lysis buffer (Buffer ATL). Moreover, the lysis is carried out at a higher temperature, but for fewer hours than the other protocol[113], making the technique time-saving. The final protocol used is further illustrated in Chapter 5 of the thesis.

CHAPTER 3:

ESTABLISHMENT OF FELINE POSITIVE CONTROLS.

3.1. INTRODUCTION.

When running PCR-based assays, having positive controls is mandatory. Having a positive control is useful, not only to check the correct amplification but also to have a reference result with which to compare the final clonal samples.

Patients diagnosed with lymphoma, could be used as positive controls and included in the routine PCR settings. For this purpose samples with the following requirements were tested: 1) histological diagnosis consistent with lymphoma; 2) known immunophenotype by immunohistochemical staining; 3) a reasonable amount of tissue, regarding sample size and preservation.

3.2. MATERIALS AND METHODS.

3.2.1 Case Selection.

The archive of the Pathology Unit of the Department of Veterinary Medicine (University of Cambridge, UK) was interrogated, and formalin fixed paraffin embedded (FFPE) tissue samples from cats diagnosed with lymphoma were selected.

Thirteen cases of lymphomas were available for analysis, but unfortunately, only in four cases, did the paraffin block contain enough tissue for analysis:

- CASE 1: case one was a Domestic Short Hair (DSH) cat diagnosed in 2015 with alimentary T cell lymphoma, intermediate to large size, involving the jejunum. Positivity to CD3 on IHC confirmed the immunophenotype. The tissue available was obtained from complete resection of the portion affected.
- CASE 2: case two was a DSH cat, diagnosed in 2012 with a diffuse gastric lymphoma of B cell lineage. Immunophenotype was confirmed by the strong positivity to CD79a on IHC. The tissue available consisted of a section measuring 10x6x6 cm, derived from surgical resection of a portion of the stomach.
- CASE 3: case three was a DSH cat diagnosed in 2016 with B cell lymphoma (confirmed by CD79a positivity on IHC) at the ileocolic junction. The sample was a section measuring 100x38x32mm containing the whole intestinal loop involved in the lymphoma.

- CASE 4: case four was a DSH cat diagnosed with B-cell mediastinal lymphoma in 2013. The tissue available was positive to CD79a on IHC and consisted of two biopsy specimens measuring 1.8x 0.1 cm and 1.2 x 0.1cm respectively.

All cases were diagnosed based on histology with Hematoxylin and Eosin staining followed by IHC using CD3 for identification of T lymphocytes and CD79a for identification of B lymphocytes. All the samples underwent an additional assessment by a Veterinary Pathologist (E.C.), to judge the cellularity and the suitability for further analysis.

3.2.2. DNA Extraction.

DNA extraction was performed on approximately 25mg of FFPE tissue, using the QIAmp Mini and Blood kit (Qiagen), according to the manufacturer's instructions, briefly described below[112].

After cutting off excess wax, the section was cut into small pieces using a sterile scalpel. The specimen was incubated with 500µL of xylene to dissolve the residual wax. Lysis was performed in two steps: first incubation with pure (96-100%) ethanol was performed; then, 20µL of Proteinase K (Qiagen) was added; finally, a specific lysis buffer provided in the kit (Buffer ATL) was added and incubation continued at 56°C for 1-3 hours, with vortexing every half an hour in order to improve tissue lysis. Treatment with RNase solution (Qiagen) at RT for 2 minutes was performed, to avoid potential interference by RNA.

Additional lysis steps were then performed: AL buffer (provided) was added to the mixture and incubated at 70°C for 10 minutes. Final ethanol addition was completed and the lysate so obtained was placed into a spin column (provided in the kit). After two washing steps (performed with buffer AW1 and buffer AW2, both supplied in the kit) final elution was performed using buffer AE (provided) after incubation at room temperature for 5 minutes. The volume of elution solution was chosen according to the tissue section available: 100µL were used for significant sections (samples 1, 2, 3) and 50µL for small sections (sample 4).

DNA was then ready for analysis or storage. Storage was performed at 4°C short term, and at -20°C for longer periods.

3.2.3. DNA Quality Assessment.

DNA quality and concentration were assessed spectrophotometrically using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific), as already mentioned[105].

We measured the quantity of DNA contained in the eluate, expressed as concentration (ng/µL). Genomic DNA extracted was considered pure if ratios $A_{260/280}$ and $A_{260/230}$ measured were ≥1.8 and ≥2 respectively.

To check the suitability of the DNA extracted to undergo further analysis Polymerase Chain Reaction amplification (PCR) of Feline Androgen Receptor (fAR) gene was performed, as suggested by Mochizuki et al.[77]:
The primer set used comprised:

forward primer: 5' CAC AAT GCC GCT TAC GGG GAC CT 3';
reverse primer: 5' AGG GGG TCA CAG ACC CTG ACT CG 3'.

PCR amplification was carried out using the TopTaq ® Master Mix kit, according to the Manufacturer's instructions[118]. The protocol is briefly described below. A mixture of 12.5µL TopTaq Multiplex PCR Master Mix, 2.5µL of 10x Coral Load Dye, one µl of 50x primer mix was prepared and loaded into a PCR tube, one per each reaction. A variable amount of eluate containing template DNA and RNase-free water was then added assuring an amount of nucleic acid around 100-120ng/µL and a total reaction volume of 25µL.

Amplification cycles included: 1) a first polymerase activation step (95°C for 5 minutes); 2) 40 cycles of denaturation (30 seconds at 95°C), annealing (90 seconds at 68°C) and extension (30 seconds at 72°C). A final extension at 68° C for 10 minutes.

Non template negative control (NTC) reactions adding RNase-free water were run in order to exclude false positive results due to contaminations of reagents.

Agarose gel electrophoresis was performed to visualise the PCR products. GelStar Stain (Lonza) was added to the 2 % agarose-TAE solution (Sigma Aldrich) during gel casting, at a concentration of 1x. For quantification of the amplicons, DNA rulers were loaded to the external wells of the gel: FastRuler Ultra Low Range DNA Ladder and FastRuler Low Range DNA Ladder (Thermo Fisher Scientific).

A total of 5 µL of sample was loaded in each well, and the samples were run at a voltage of 80V for 60 minutes. Gels were scanned using the Gel Doc™ XR+ (BioRad) scanner, and the excitation and emission were set at 493 nm and 527 nm respectively, according to the stain requirements[119].

Feline DNA was considered suitable for further analysis if the reaction yielded a single distinct band of around 189 bp[77].

3.2.4. Polymerase Chain Reaction (PCR).

3.2.4.1. Primer set.

Primers previously published[77,78,122] were purchased (Eurofins Genomics). The primers were mixed to obtain a final concentration of 100pmol/µL, and a 50x primer mix was used.

B cell clonality was assessed by the detection of rearrangement of IgHV1 and IgHV3 as previously described[77,122]. Three primer sets were annealed to framework region 2 (FR2- namely the V3F3 mix) and framework region 3 (FR3- namely V1F2 and V3F4 primers) of the variable (V) region genes were used in conjunction with five consensus primers to the 3′ ends of the joining (J) region.

The use of one primer mix detected T cell clonality[78]. The five forward primers were placed on the V regions (namely V1-2, V3, V4 and V5) in conjunction with three reverse primers, located in the J region (J1, J2, J3).

Sequences used for amplification and expected band size are listed in **Table 6**.

3.2.4.2. Reaction settings.

The amplification was performed in triplicates for all the samples.

The reactions were carried out using the Type-it ® Mutation Detect PCR kit, according to the Manufacturer's instructions[117]. Briefly, A mixture of 12.5µL Type-it Multiplex PCR Master Mix, 2.5µL of Q solution, 2.5µL of Coral Load Dye 10x, and 1µl of 50x primer mix was loaded in individual PCR tubes. A variable amount of eluate containing template DNA and RNase-free water was added, in volumes assuring 100-120 ng/µL of gDNA and a final reaction volume of 25µL per tube.

Negative control reactions adding RNase-free water were run in order to exclude false positive results due to contaminations of reagents.

TARGET GENE		NAME	TARGET REGION		SEQUENCE	EXP SIZE (bp)	REF
Feline TCRG	Forward primers	TCRγ/V1-2	*TCRG-V1-2*	CDR3	5' GGS AGA AGA GCG ACG AGG GCG TG 3'	80-120	Mochizuki et al. 2012[78]
		TCRγ/V3	*TCRG-V3*		5' GGG CGA AGA GCG ATG AGG GAG TG 3'		
		TCRγ/V4	*TCRG-V4*		5' GTA GTG AGG AGR ATG CTG GTC TG 3'		
		TCRγ/V5	*TCRG-V5*		5' GGC AGA AGC ATG ACA AGG GCA TG 3'		
	Reverse primers	TCRγ/J1	*TCRG-J 1*	J	5' CCT GAG CAG TGT GCC AGS AC 3'	Mochizuchi et al 2012[78]	
		TCRγ/J2	*TCRG-J2*		5' GGG GGA GTT ACK ATG ASC TTA RTT CC 3'		
		TCRγ/J3	*TCRG-J3*		5' ATC CAG ATC TCA GGT TTG GGA GGA GG 3'		
Feline IGHV	Forward primers	V_1F_2	*IGHV1*	FR3	5' GCA GAC ACA TCC ACA AAC ACA GCC TAC 3'	100-170	Mochizuki 2011[77]
		IgH2	*IGHV3*	FR 2	5' CCA GGC TCC AGG GAA GGG 3'	250-300	Werner2005[122]
		V_3F_3			5' GGG TCC GCC AGG CTC CAG G 3'	210-280	Mochizuki 2011[77]
		IgH3		FR 3	5' TCC AGA GAC AAC GCC AAG AAC 3'	130-180	Werner2005[122]
		V_3F_4			5' GGC CGA TTC ACC ATC TCC AGA GAC 3'	120-190	Mochizuki 2011[77]
	Reverse primers	J1	*IGHJ*	J	5' ACA CCG TCA CCA GGG CTC C 3'	Werner2005[122]	
		J2			5' TGA GGA CAC TGT GAC TAT GGT TCC 3'		
		JD			5' GGA CAC CGT CAC YAK GVY TCC 3'		
		JR1			5' GCY STC ACC AGG RYT CCY BGGC-3	Mochizuki 2011[77]	
		JR2			5' GCT GYG ACH MTD GTT CCA YGG CCC 3'		
		JR3			5' GCG RTG AYC WGG GTR YCY TGG C 3'		
		JR4			5' GCG GTG ACC AGG GTC CCG GGG CCC 3'		
		JR5			5' GCC GTC ACC AGG GTT CCG ACG CC 3'		

Table 6. Primers used for standard PCR amplification of feline TCRG and IGH loci.establishment of Positive controls

3.2.4.3. Cycling Protocols.

The same cycling conditions were employed for all the primer sets used.

Activation of the Polymerase at 95° C for 5 minutes was followed by forty cycles of denaturation (95°C for 30 seconds), annealing (68°C for 90 seconds) and elongation (72 °C for 30 seconds). A final extension at 68°C for 10 minutes was then performed.

All the samples were finally denatured at 95°C for 5 minutes and allowed to reanneal at 4 °C for one hour (heteroduplex analysis). This step was performed to increase the sensitivity of the PCR and to minimise the possibility of misdiagnosis by pseudoclonality results[123].

The reactions were performed using a T 100 Thermal Cycler (BioRad).

Known positive controls included DNA extracted from two cultured cell lines one for B cell and one T cell (MS-4[124] and FT-1[125]). These were run as positive controls on all gels.

Negative control reactions adding RNase-free water were run in order to exclude false positive results due to contaminations of reagents.

3.2.5. VISUALISATION OF PCR PRODUCTS.

Two different techniques were used for the detection of the amplicons.

3.2.5.1. Agarose Gel Electrophoresis.

Low Melting Point (LMP) Agarose powder (Sigma) was dissolved in Tris-acetate-EDTA buffer (TAE) at a concentration of 4%. The mixture was then heated in the microwave oven until complete dissolution. Staining was performed on pre-cast gels using GelStar at a final concentration of 1x: stain was added to the liquid agarose at a temperature of 65/75°C. The mixture was then gently poured into the electrophoresis platform and allow to solidify.

When ready the gel was placed into the electrophoresis chamber filled up with TAE buffer.

Five µL of PCR products were then loaded into each well of the gel. The electrophoretic run was conducted at 200 V for 5 minutes. Voltage was then lowered to 160 V, and the run was considered completed after 45 minutes.

DNA rulers were loaded to the external wells of the gel: FastRuler Ultra Low Range DNA Ladder and FastRuler Low Range DNA Ladder (Thermo Fisher Scientific).

After the run was completed, gels were scanned using the Gel Doc™ XR+ (BioRad) scanner. Excitation and emission wavelength was set at 493nm and 527nm respectively, according to the stain requirements[119].

3.2.5.2. Polyacrylamide Gel Electrophoresis (PAGE).

Vertical Polyacrylamide gels (Novex TBE Gels, 20%, ThermoFisher Scientific) were used as the second electrophoresis method.

The gels are located into plastic cassetteee and were stored at 4°C until use. After removing the cassette from the pouch and a quick wash with deionised water, the tape present on the bottom of the cassette and the plastic comb were gently removed. The wells were then rinsed with TBE buffer.

The run was conducted using the XCellSureLock™ Mini-Cell Electrophoresis Chamber (ThermoFisher Scientific), and the gels were placed individually on the bottom of the chamber, filled with TBE buffer.

Samples were prepared by mixing 5µl of the PCR products with two µL of 5x Novex™ Hi-Density TBE Sample Buffer (ThermoFisher Scientific) and deionised water according to the manufacturer's instructions.

A volume of 6µL of the mixture was loaded onto the gel.

DNA rulers (FastRuler Ultra Low Range DNA Ladder and FastRuler Low Range DNA Ladder Thermo Fisher Scientific) were also prepared as previously described and loaded onto the gel.

The run was conducted at a voltage of 180 for 90 minutes until the bromophenol blue contained in the sample buffer (dark blue colour) reached the bottom of the gel.

Staining was performed afterwards, using a 1x solution of GelStar (Lonza) in TBE buffer. After opening the cassette, the solution was gently poured on the gel, which was consequently incubated for 30 minutes in the dark[119].

Finally, gels were scanned using the Gel Doc™ XR+ (BioRad) scanner. Excitation and emission wavelength was set at 493 nm and 527 nm respectively, according to the stain requirements[119].

Visualisation of amplicons of the expected size according to the reaction performed (**Table 6**) was suggestive of a clonal result. The presence of smeary bands or no visible bands in the expected area of the gel was supportive of a polyclonal or negative result. Pseudoclonal results were given by the visualisation of the expected sized band in just one of the duplicates.

Table 7. Extraction data from the four cases selected as feline positive controls.

Sample ID	DNA Concentration (ng/ul)	Absorbance ratio ($A_{260/280}$)	Absorbance ratio ($A_{260/230}$)
Case 1 (CD3+)	34.24	2.04	2.28
Case 2 (CD79a+)	151	2.04	1.82
Case 3 (CD79a+)	219.26	2.05	1.74
Case 4 (CD79a+)	20.05	2.01	1.63

3.3. RESULTS.

All the samples yielded good concentrations of gDNA and the fAR gene was successfully amplified in all the samples (**Table 7**).

Rearrangement of the feline IgHV3 was investigated with four primers targeting FR2 (IgH2 and V_3F_3)[77] and FR3 (IgH3 and V_3F_4[77]). All the samples gave similar results both on Agarose gel electrophoresis and PAGE, thus will be illustrated concomitantly.

Case no.4 showed clonal rearrangement of the IgH2 primer, both on agarose and PAGE gels. The presence of a bright band measuring approximately 250-300bp was suggestive of clonal rearrangement in the FR2 of the IgHV3 gene, observed in B cell clonal populations.

The primer V_3F_3 gave a faint band of approximately 210/280bp in cat no.4. This result was confirmed on PAGE, and the band appeared more prominent on gel.

The primer IgH3 gave clonal amplicons measuring 130-180 bp in cat no.4 visible both in the agarose and in the PAGE gel.

Finally, primers V_3F_4 and V_1F_2 were tested both in singleplex reaction. V_3F_4 primer gave a clonal result in cat no.4, and clonality was confirmed by PAGE, with the visualisation of a neat band of 120/190 bp. No clonal result was retrieved by V_1F_2 primer from any of the sample.

Clonal rearrangement of the feline TCRG[78] locus was evident in cat no.1 yielding a clear band visible both in the agarose and in the PAGE gel of approximately 80-120 bp. Unfortunately, the result was reproducible on two of the three PCR triplicates. Cat no.3 on agarose showed a bright band, and interpretation was not straightforward: however, PAGE showed the result as polyclonal, removing any doubt of clonality.

Images from PAGE analysis of V_1F_2,V_3F_4 and TCRG reaction, unfortunately, are not available.

We selected the sample from cats 1 and 4 as positive controls for B cell and T cell PARR, given the concordance between the immunophenotype and the PARR result.

All the results are shown in **Figure 10** and **Table 7**.

Figure 10. Visualisation of PCR amplicons on gels. NTC and positive cell line FT-1 were run along with the samples (not. shown). Size rulers are run along with the samples in all the gels.

Figure 10A. TAE- 4% LMP Agarose gel, patient no. 4, a prominent monoclonal band of approx. 200 bp for IGH gene rearrangements detected by primer V_3F_3 and IgH2; Smaller prominent monoclonal bands are evident for V3F4 and IgH3 reaction (approx..120/190bp);

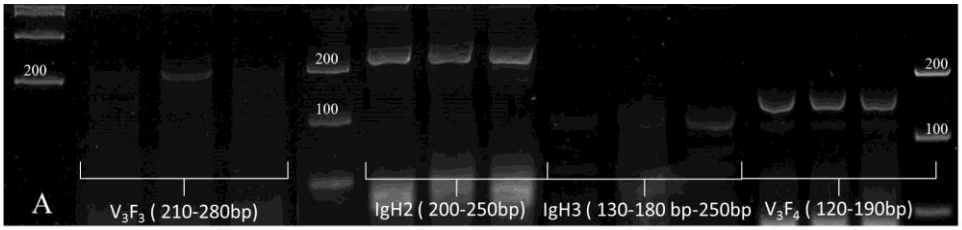


Figure 10B. TBE 20% PAGE gel, patient no. 4; a prominent monoclonal band of approx. 200 bp for IGH gene rearrangements detected by primer V3F3 and IgH2; Smaller prominent monoclonal bands are evident for the IgH3 reaction (approx.120/190bp);

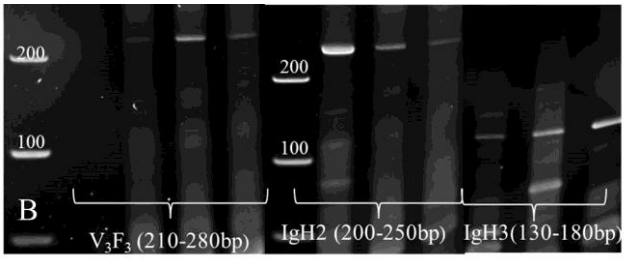
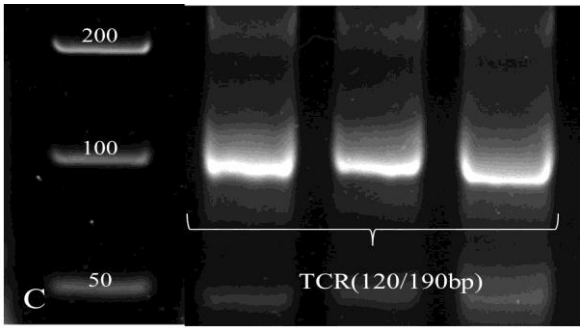


Figure 10C. TAE- 4% LMP Agarose gel, patient no.1, a prominent monoclonal band of approx. 100 bp for TCRG gene rearrangement detected by primer TCR.



3.4. DISCUSSION

With this study, we were able to select suitable gDNA samples to use as positive controls. Unfortunately, we did not perform any sequencing of the products obtained by PCR amplification, to detect with precision the clonal rearrangement of the neoplastic cells analysed. However, the concordance between IHC and PARR was convincing for the further use of these specimens as positive controls.

Moreover, we included in our study DNA extracted from two cell lines (MS-4[124] and FT-1[125]) kindly provided by Dr Hammer, from the University of Veterinary Medicine of Vienna (Austria). Cell lines derived DNA was run along with DNA extracted from our samples, to have a second reference sample for our PCR reactions.

Two cats (cat no.2 and cat no.3) did not show any clonality for any of the primer sets used, despite the confirmed B immunophenotype on IHC.

This result can be explained by the presence of too degraded DNA[63] obtained from FFPE tissue. It is possible that given the age of the block (the case2, in particular, was processed in 2012, six years before DNA extraction) older than the other samples, despite the excellent index of quality given by spectrophotometry, the DNA was not an integer. Thus, the integrity of gDNA should be checked first to adapt the concentration used in the Ig/TCR assays. Moreover, the presence of PCR inhibitors could impair satisfactory amplification results. In this case, however, the PCR amplification of the control gene gave positive results, confirming the suitability of the DNA for PCR.

Another reason for impairment of clonality could be attributable to the presence of only very few neoplastic cells[63]. This event could be excluded in the present case, as a Veterinary Pathologist checked the samples before analysis.

For this reason, it is more likely that the primers tested didn't cover all the possible gene rearrangements, which is a possible event, especially in B cell neoplasia. In fact, Somatic Hypermutation (SHM) is a mechanism which triggers antibody diversity and occurs after the antigenic stimulation of B cells[126]. Stepwise incorporation of a single nucleotide into

the V gene of both Light and Heavy chains can impair the gene coverage of the primer used.

3.5. CONCLUSIONS.

To summarise we selected:

Cat no.4 as a positive control for primers B cell clonality investigated with the primers: IgH2, IgH3, V_3F_3 and the primer mix V_1F_2/V_3F_4.

Cat no.1 as a positive control for primer TCR.

CHAPTER 4:

CANINE CLONALITY ASSAY

4.1. AIMS OF THE STUDY.

The primary aim of this study was to refine the diagnosis of canine leukaemia achieved by flow cytometry (FC) using PCR techniques (PARR).

This refined PCR method would then be compared to FC in the same set of subjects.

Both methods will be compared in dogs with lymphoproliferative disease and non-malignant haematological proliferative conditions.

These aims are based on the following:

1) The sensitivity of PARR in detecting a clonal population within a reactive lesion,
2) The importance of immunophenotype in lymphoma/ leukaemia diagnostics,
3) The study will be specifically aimed at refining the methods for PARR in the canine species.

4.2. MATERIALS AND METHODS.

4.2.1. Case Selection.

The archive of the Central Diagnostic Service of the Department of Veterinary Medicine (University of Cambridge) was interrogated, in order to select cases of absolute lymphocytosis. Twenty-nine cases were selected from July 2015 to January 2018, divided into two groups: (a) dogs diagnosed with leukaemia by morphological assessment and confirmed by FC (nineteen); (b) dogs diagnosed with reactive leucocytosis (ten), due to evidence of inflammatory conditions (such as high C reactive protein levels, or a recent history of vaccination or immune-stimulation), not attributable to any neoplastic disease. The latter group was not submitted for immunophenotyping, as there was no morphological evidence of neoplastic disease by microscopic evaluation.

A CBC was run on a Sysmex XV 1000 which included a differential count which was checked manually. The laboratory has established RI for this instrument. Cell

morphology was examined by a Clinical Pathologist to determine if the sample was suitable for FC.

4.2.2. Flow Cytometry.

Flow cytometry was performed using a panel of antibodies directed to the membrane and intracellular proteins known as "clusters of differentiation" (CD). The different expression patterns, together with clinical presentation and microscopic assessment, allowed determination of the cell lineage undergoing neoplastic proliferation.

The antibody panel includes:

- STEM CELL MARKERS, expressed by the early precursor of hematopoietic cells. In malignancies, their expression is strictly dependent on the level of undifferentiation of the proliferating neoplastic cells.
- COMMON LEUKOCYTE MARKERS, expressed on leukocytes, both myeloid and lymphoid.
- LYMPHOCYTE MARKERS expressed mainly on lymphocytes. Expression of a single marker or combination of different markers allows distinguishing T-cells (with T helper and T cytotoxic cells identifiable) and B-cells.
- MYELOID MARKERS: Mainly expressed by the myeloid lineage.
- OTHERS: they can be expressed both by lymphoid and myeloid cells. Their significance is sometimes unknown, and they represent a mutual aid in the diagnosis of lympho or myeloproliferative neoplasia.

The antibody panel used, with patterns of expression is shown in **Table 8**.

Blood was aliquoted into tubes to give a cell count of 1x 10^6/L cells per tube. The volume of the sample was based on the total WBC count obtained from the Sysmex analyser. Antibodies were added at dilutions previously determined and standardised by the laboratory for use in the dog leukaemia panel. Cells with the antibodies or the isotype controls were incubated for 20 minutes in the dark. If the antibody was not directly conjugated to the fluorochrome, an additional incubation with a secondary antibody was

performed. Red blood cells were lysed using a solution of Ammonium Chloride. To eliminate excess antibody and debris, several washing steps were performed. For the intracellular antibodies (CD3-12 or CD79a), an initial step of fixation and consequent permeabilisation was performed with Fix and Perm (Caltag Laboratories). Finally, cells were resuspended in 200µL of PBA.

Each tube was analysed with a BD Accuri C6 Plus cytometer, and BD Accuri C6 Plus Software.

Cells of interest were gated according to Forward Scatter (FS) and Side Scatter (SS) properties. Once the population is gated, the antibody expression profile is expressed as a scatter plot, which correlates with the fluorescence intensity detected for each fluorochrome. Isotype controls were set as negative controls. Examples of scatter plots are shown in **Figure 11.**

After analysis excess samples were archived and stored at -80° C until further analysis.

Figure 11. Example of a FC scatterplot. In the first graph, the population of interest is gated within the total population analysed (circled in red). Cells (each represented by the dots in the plot) are displayed according to the FS (indicating the size of the cell) and the SS (indicating the complexity of the cytoplasm). In the case shown cells are probably big with low complexity (resembling intermediate/large lymphocytes). The second plot shows the isotype control for the mAb CD34 and MHCII. Isotype control is a negative control. In the third graph positivity for CD34 (70,4% of the gated cells), whereas concurrent reactivity to MHCII is not present.

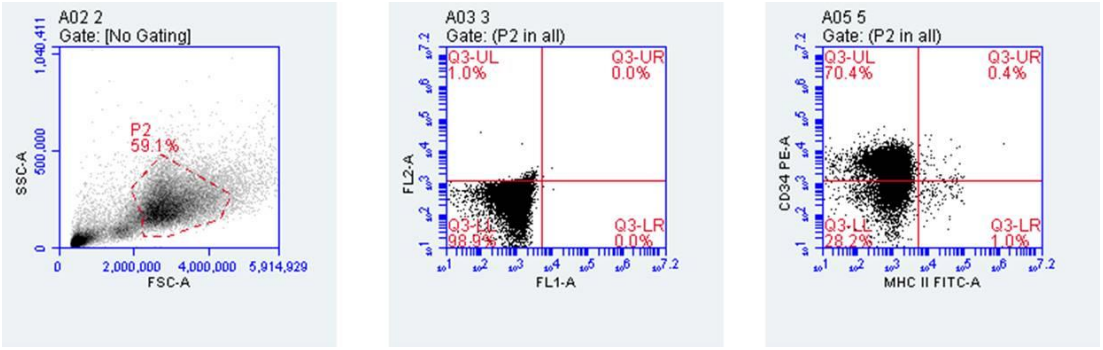


Table 8. Details of the Monoclonal antibodies (mAbs) panel used in the study for Flow Cytometry immunophenotyping in the dog.

MARKER OF		ANTIBODY NAME	CLONE	COMPANY	TARGET	conjugated with	ISOTYPE CONTROL
STEM CELL		CD34	1H6 (RUO)	Pharmigen™	Hematopoietic progenitors (other lineage progenitors)[39,127–129]	PE	IgG2a
		CD117	ACK45	Pharmigen™		FITC	Rat IgGb: FITC
PANLEUKOCYTES		CD 18	CA1.4E9	Bio Rad	Panleukocyte[39]	AlexaFluor® 647	mouse IgG1: AlexaFluor® 647
		CD45	YKIX716.13	Bio-Rad		APC	rat IgG2b: APC
		MHCII	YKIX334.2	Bio-Rad		FITC	IgG1
LYMPHOCYTES	T	CD3	CA17.2A12	Bio-Rad	T Lymphocytes[39]	FITC	IgG1: FITC
		CD3 (intracellular)	CD3-12	Bio Rad		FITC	Rat Igg1: FITC
		CD5	YKIX322.3	Bio Rad	T Lymphocytes[39], Thymocytes, NK cells[130,131]	FITC	IgG2a: FITC
		CD8	YCATE55.9	Bio Rad	T Lymphocytes (cytotoxic)[39,131]	AlexaFluor® 647	rat IgG1: AlexaFluor® 647
		CD 4	YKIX302.9	Bio Rad	T Lymphocytes (helper)/neutrophils[39,131,132]	PE	rat IgG2a:PE
	B	CD21	CA2.1D6	Bio Rad	B Lymphocytes (mature)[133]	PE	mouse IgG1:PE
		CD79a	HM57	Bio-Rad	B Lymphocytes[39]	PE	IgG1
MYELOID		CD14	TÜK4	Bio Rad	Monocytes/Macrophages[134,135]	FITC	IgG2a:FITC
		anti-neut	NA	NA	Neutrophils	FITC	rabbit F(ab) anti mouse IgG:FITC
		MPO	2C7	Bio Rad	Neutrophils[39,134,135]	FITC	IgG1
OTHERS		CD11d	CA11.8H2	Bio Rad	Subset of macrophages[136],monocytes[135,137] CD8+ Tcells, splenic pulp γ/δ T cells, LGLs	FITC	mouse IgG1+ rabbit F(ab')2 anti-mouse IgG:FITC
		CD90 (Thy-1)	YKIX337.217	Bio Rad	T lymphocytes[134,135,138]	rabbit F(ab) anti rat IgG:FITC	Rat Igg2b+rabbit F(ab) anti rat IgG:FITC

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4.2.3. Clonality Assay.

4.2.3.1. DNA Extraction.

The archived samples were thawed at room temperature for 30 minutes and, when completely defrosted, were mixed by gentle vortexing.

Genomic DNA was extracted using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) according to the protocol established in Chapter 2.

Volume containing $9x10^6$ cell/L was considered suitable for optimal DNA extraction. To reach the volume recommended by the manufacturer, small samples were pooled in PBS to reach the volume of 200µL. If, on the contrary, the volume required was >200 µL, a cell pellet obtained by centrifugation was resuspended in 200µL of PBS.

Volumes and Resuspension Solutions are listed In **Table 9**.

Lysis was performed by incubating the sample with 20µL of Proteinase K (Sigma, ≥30units/mg protein) and 200µL of Lysis Solution C (provided with the kit) at 55°C for 10 minutes. Ethanol was added to the sample to optimise DNA binding capacity to the column. The lysate so obtained was placed onto the Spin Columns (provided in the kit) and centrifuged at 13000 rpm for 30 seconds, allowing the DNA to strongly bind while the excess solution flowed through the membrane. Two washing steps were carried out to reduce contamination by proteins or polysaccharides.

Elution of the DNA from the column was performed by addition of 150µL of elution buffer (provided) followed by incubation for 5 minutes at room temperature to promote an optimal yield. The final centrifugation allowed collection of the DNA eluate in a collection tube.

DNA was stored at 4°C short term (1-2 weeks) or -20 C (1-2 months) until analysis.

Table 9. Details of dog blood samples used for DNA extraction for PARR analysis. Volumes used for DNA extraction in order to obtain $9x10^6$ cells/Lin a final volumes of 200µL. **R,** reactive lymphocytosis; **N**, neoplastic lymphocytosis.

Sample ID	Immunophenotype	WBC($x10^9$/L)	Volume (µl) required for WBC=$9x10^9$/L	Resuspension solution (µL)	tot volume (µL)
A	R	14.7	612.2		200
B	R	60.67	148.3	51.7	200
C	R	16.06	560		200
D	R	31.45	286.2		200
E	R	19.21	468.5		200
F	R	67.16	134	66	200
G	R	18.59	484.1		200
H	R	11.09	811.5		200
I	R	34.56	260.4		200
J	R	20.09	448		200
1	N	254.19	35.4	164.6	200
2	N	434.51	20.7	179.3	200
4	N	25.9	347.5		200
5	N	71.2	126.4	73.6	200
6	N	94.83	94.4	105.1	200
7	N	18	500		200
8	N	116.09	77.5	122.5	200
9	N	57.66	156.1	43.9	200
10	N	14.06	640.1		200
11	N	86.05	104.6	95.4	200
12	N	279.85	32.2	167.8	200
13	N	15.79	570		200
14	N	77.92	115.5	84.5	200
16	N	13.40	548.8		200
17	N	75.9	118.6	81.4	200
19	N	11.04	815		200
20	N	54.89	164	36	200
21	N	11.52	781.3		200
22	N	28	321.4		200

4.2.3.2. DNA Quality Assessment.

Extracted genomic DNA was analysed using a Nanodrop1000 (ThermoFisher Scientific) spectrophotometer. Spectrophotometry provides quantification of DNA in the eluate (expressed as ng/µL) and information about protein (calculating the ratio between 260nm and 280nm absorbance, $A_{260/280}$) and phenol or salt (ratio between 260 and 230 absorbances, $A_{2607230}$) contamination[105].

Two µL of eluate were pipetted directly onto the measurement pedestal. The sampling arm was then lowered and the sample was maintained in place between the two optical fibres by the surface tension. The corresponding elution solution was used as a blank prior to each analysis. DNA concentration was expressed as ng/µl. DNA showing $A_{260/280}$ around 1.8 and $A_{260/230}$ around 2.0 was consider of high purity.

Normalization of DNA was performed before setting up a polymerase chain reaction amplification (PCR) for the juxtamembrane region of the canine C-kit gene, as suggested by Tamura et al[67].
The sequence targeting exon 11 was used:

forward primer: 5' CCC ATG TAT GAA GTA CAG TGG AAG 3';
reverse primer: 5' GTT CCC TAA AGT CAT TGT TAC ACG 3'.

PCR amplification was carried out using the Type-it ® Mutation Detect PCR kit, according to the manufacturer's instructions[117]. The protocol is briefly described above. A mixture of 12.5µL Type-it Multiplex PCR Master Mix, 2.5µL of Q solution, 2.5µL of 10x Coral Load Dye, 1µl of 50x primer mix and a variable amount of eluate containing template DNA and RNase-free water was loaded in individual PCR tubes. Despite the stability of the Polymerase contained in the kit (HotStarTaq Plus) all the reactions were conducted on ice. The volume of eluate added assured a total amount between 100-120ng/µL of DNA per reaction, and the water was added in order to reach a total volume of 25µL per tube.

After a first activation of the polymerase by heating the sample at 95°C for 5 minutes, Thirty-five cycles of denaturation of the DNA (30 seconds at 95°C), annealing (60 seconds at 64°C) and extension(1 minute at 72°C) were performed.

Agarose gel electrophoresis was performed to visualize the PCR products. GelStar Stain (Lonza) was added to the 2 % agarose gels during gel casting, in a concentration of 1x. For quantification of the amplicons, DNA rulers were loaded to the external wells of the gel: FastRuler Ultra Low Range DNA Ladder and FastRuler Low Range DNA Ladder (Thermo Fisher Scientific). A total of 5μL of sample was loaded in each well and the samples were run at a voltage of 80V for 60 minutes. Gels were scanned using the Gel Doc™ XR+ (BioRad) scanner. The excitation and emission were set at 493 nm and 527 nm respectively, according to the stain requirements[119].

The reaction was considered positive if a single discrete band weighing approximately 200bp[67] was visible on the gel.

In conclusion, the combination of the aforementioned information (good concentration, $A_{260/280}$, $A_{260/230}$) and the visualisation of the band corresponding to the C-kit gene, confirmed the suitability of the examined DNA for further analysis.

4.2.3.3. PCR for Antigen Receptor Rearrangement (PARR).

The clonality assay was directed to amplify the genes encoding for antigen receptors of B (heavy chain of surface immunoglobulins- IGH) and T (γ chain of TCR- TCRG) lymphocytes. These genes are composed of four different regions: the Variable (V), the Joining (J) the constant (C) and the Diverse (D).

In the current study, primers directed to the V and J regions of TCRG and of the IGH were used.

Primers previously published[72,79] were purchased (Eurofins Genomics). The primers were mixed in order to obtain a final concentration of 100pmol/μL and A 50x primer mix solution was prepared.

B cell clonality was assessed using two primer sets annealing framework region 2 (FR2) and FR3 of the variable (V) region genes were used in conjunction with two consensus primers to the 3′ ends of the joining (J) region[79]. These primers were conventionally named IgH2 and IgH3 respectively.[79]

T cell clonality was detected by the use of two primer sets. The first one, designed and tested by Valli et al.[79], included a single forward primer and a single reverse primer, directed to the V region of the CDR3. This primer was called TCRG. The second one was designed as a multiplex PCR in order to cover as many regions of the TCRG as possible at once. Forward primers were set at three V regions (namely V2, V3 and V 7); Reverse primers were instead place on the J region, for a total of 6 different primers targeted to different genes.[72] This primer was named STCRG.

Primer sets and expected band size for each reaction are shown in **Table 10**.

Table 10. Primer sets used for amplification of Canine TCRG (two primers: TCRG[79] and STCRG[72]) and Canine IGH (two primers: IgH2 and IgH3)[79]. The expected size of the PCR products are shown accordingly.

		NAME	TARGET gene	TARGET region	SEQUENCE	EXP SIZE	REF
Canine TCRG	forward	TCRG	TCRG-V	*FR3*	5' TGK TGC AGA ARC TGG AGA AGA 3'	111 bp	Valli et. Al (2006)[79]
	reverse		TCRG-J	*FR3*	5' GCA CTG TGC CAG GAC CAA ATA 3'		
	forward	STCRG	V 2-1		5' GAA GGC GTG TAC TAC TGC GCT G 3'	95-150 bp	Keller et al. (2012)[72]
			V 2-2		5' GAG GGC GTG TAC TAC TGT GCT G 3'	165-205	
			V 3-1		5' TGT TAA GGA AAC AAG ATG AGG CCA 3'	80-140	
			V 3-2		5' TCT TAA GGA AAC AAC ATG AGG CTG TG 3'	150-200	
			V 7-1		5' AAG TAA AAA TGC TCT TAC TTC CAC TTC AAC 3'	140-185	
			V 7-2		5' GTA AAA ATG CCG TTA CTT CCA CAT CAA CTT 3'	90-140	
			Ja (J 1-2)	*J1-2, J2-2-, J3-2, J4-2, J5-2, J7-2*	5' TTG TGC CAG GAC CAA ACA CTT T 3'		
			Jb (J 6-1)	*J6-1, J8-1*	5' GGG GAG TTA CTA TGA GCT TAG TTC CTT 3'		
	reverse		Jc (J 3-1)	*J3-1, J4-1*	5' GAG GAG TTA CTA TAA GCC TAG TAC CTT CTG 3'		
			J 2-1		5' GAG GAG TTA CTA TAA ACC TGT TAA CTT CTG 3'		
			J 5-1		5' GGG GAG TTA CTA TGA GAT TAG TTC CTT CGT 3'		
			J 6-2		5' GTG TGT CAG GAC CCA TCA CTT TGT T 3'		
Canine IgH	forward	IgH2	Ig VH	- *FR 2*	5' GGA ARG GKC TRC AGTGGG T 3'	250 bp	Valli et al. (2006)[79]
		IgH3	Ig VH-	*FR 3*	5' GMC GVT TCA CCA TCT CCA RRG 3'	180 bp	
	reverse		IgHJ	*J*	5' TGA RGA GAC RGT GAC CWG GGT 3'		
			IgHJ	*J*	5' GGA CAC GAA GAS TGA GGT GCC 3'		

The reactions were carried out using the Type-it ® Mutation Detect PCR kit, according to the manufacturer's instructions. Briefly, A mixture of 12.5µL Type-it Multiplex PCR Master Mix, 2.5µL of Q solution, 2.5µL of Coral Load Dye 10x, 1µl of 50x primer mix solution loaded in individual PCR tubes, one for each sample to analyse. The volume of eluate added assured 100-120 ng of DNA, and a final reaction volume of 25µL was reached adding RNAse free water accordingly.

Cycling conditions for IgH2, IgH3 and TCRG primers[79] were designed as a touchdown protocol in order to increase specificity of the reactions. Activation of the Polymerase at 95° C for 5 minutes was followed by five cycles of denaturation (94° C for 30 s), annealing (72°C for 90 s); five cycles of denaturation (94°C for 30 s) and annealing (70°C for 90 s); and finally, 35 cycles of denaturation (94°C for 30 s) and annealing (68°C for 90s). A final extension at 68°C for 10 minutes was then performed.

Cycling conditions for the STCRG primer[72] were slightly different. The initial step of Polymerase activation was maintained and followed by 35 denaturation (95 °C for 30 sec), annealing (64°C one minute) and extension (72°C one minute) cycles. Final extension was performed at 68°C for five minutes.

Heteroduplex analysis (a final denaturation step and reannealing at 95°C for 5 minutes and 4 °C respectively) was performed. This step aims to increase the specificity in recognition of true monoclonal out of polyclonal populations.[123,139]

All the cycles were performed using a T 100 Thermal Cycler (BioRad).

Each reaction was conducted, in duplicates in order to detect pseudoclonality; moreover, positive (clonal) and negative controls were run along with the patients samples; DNA from patients previously diagnosed with B or T cell neoplasia were used as clonal controls. Negative reactions were conducted using DNA free water instead of gDNA.

4.2.3.4 Visualisation of PCR products.

Clonality was detected by visualisation of the amplicons on LMP agarose gel after electrophoresis.

Briefly, the LMP Agarose powder was dissolved in Tris acetate-EDTA buffer (TAE) at a concentration of 4%. The mixture was then heated in a microwave oven until complete dissolution of any clots or bubbles. Staining was performed on pre-cast gels using GelStar Stain (Lonza) at a final concentration of 1x: stain was added to the liquid agarose at a temperature of 65/75°C. The mixture was then gently poured into the electrophoresis platform and allow to solidify.

When ready the gel was placed into the electrophoresis chamber filled with TAE buffer.

The PCR products (5µL) were then loaded into the wells of the gel. Electrophoresis was conducted at 200V for 5 minutes. Voltage was then lowered to 160V, and the run was considered completed after 45 minutes.

DNA rulers were loaded to the external wells of the gel: FastRuler Ultra Low Range DNA Ladder and FastRuler Low Range DNA Ladder (Thermo Fisher Scientific).

After runs were completed, gels were scanned using the Gel Doc™ XR+(BioRad) scanner. Excitation and emission wavelengths were set at 493nm and 527nm respectively, according to the stain requirements[119].

The reaction was considered positive if one single or multiple bands of the expected size were visualised (according to the reaction performed, **Table 10**) and was suggestive of a clonal result. The presence of smeary bands or poorly visible or no bands in the expected area of the gel was supportive of a polyclonal or negative result. Pseudoclonal results were given by the visualisation of an expected sized band in just one of the reactions run in duplicate for the same sample.

4.3. RESULTS.

4.3.1. CASE DESCRIPTION.

The first group consisted of four (14%) females and twenty-four (83%) males. Gender was not known in one dog. The most commonly encountered breed was Labrador Retriever (six individuals) and Retrievers (not otherwise specified, three cases). Other breeds were represented by a sole individual (Crossbreed, Boxer, Golden Retriever, Jack Russel Terrier, Shih-Tzu, Pointer, Cocker Spaniel, Weimaraner, Irish Setter, Cavalier King Charles Spaniel). Median age at presentation was eight years (minimum two years, maximum thirteen years).

Vague and non-specific symptoms were the most commonly encountered: lethargy (seven cases); pyrexia (four cases), anorexia (two cases), exercise intolerance (one case). Enlargement of the internal organs was also a common sign, with mild lymphadenopathy (three cases) being the most encountered, followed by splenomegaly (four cases) combined with hepatomegaly in one dog, and evidence of a mediastinal mass in one individual. Some of the cases were referred for suspicious CBC alteration such as lymphocytosis (three cases), or evidence suggestive of bone marrow involvement (pancytopenia and anaemia in one case each category). Other systems involved were gastrointestinal tract (with vomiting and diarrhoea in one case), joints (with lameness in two cases) and respiratory (with two dogs coughing in the absence of a mediastinal mass). (**Table 1S**, Supplementary Information).

Haematological findings were available for all the dogs enrolled. Median WBC was 57.66 $x10^9$/L, with a maximum 434.51$x10^9$/L and a minimum 11.04 $x10^9$/L. High WBC were evident in fourteen cases (median 76.91 $x10^9$/L). Differential leukocyte counts were available in eleven dogs: of these, seven showed neutropenia and one neutrophilia. Eight dogs showed lymphocytosis: of the three remaining, two (case 8 and 5) showed lymphopenia but had high automated counts of atypical cell. One dog (case no.7) had a normal lymphocyte count. Monocytes were high in two cases (case no.17 and no.1), low in three (case no. 4, 5, 8) and within the normal range in the rest of the cases. Only two cases had high eosinophil counts, with an extremely high count in one case (case no.1, count of 74.29$x10^9$/L). Anaemia was evident in most of the cases (low RBC in 89.5% of the cases, low Hb and low HCT in 84.2% of cases). Automated platelet counts and microscopic evaluation were available in twelve

cases: thrombocytopenia was evident in five cases, whereas normal counts were registered in the remaining seven cases. (**Table 2S**, Supplementary Information).

Detailed information on cell differential counts was not available for many of the cases due to the presence of high numbers of atypical cells in the samples which were not classified by the haematology analyser. Inspection of all blood smears confirmed the presence of these atypical cells. However, many early-stage cells were difficult to classify on morphology alone and in a manual differential were reported as "atypical".

Dogs belonging to the second group had little information about clinical history. However, information about signalment was available for all the ten dogs enrolled. Gender was equally distributed in the group, and breed distribution varied (three cocker spaniels, and one individual for Basset Hound, Flat Coated Retriever, Labradoodle, Lhasa Apso, Pug, Rhodesian Ridgeback, Schnauzer). Median age was two years. Age was not known in one case. Complete blood cells count was available in all the cases: all the cases showed lymphocytosis (median $6.7x10^9$/L). Neutrophilia was evident in seven cases (median count $23.62x10^9$/L) and monocytosis in three cases (median count $2.83x10^9$/L). Eosinopenia was the most encountered eosinophils alteration, involving seven cases out of ten. No atypical cells were recognized in the automated counts. (**Table 3S**, Supplementary Information).

Detailed signalment, clinical presentation and haematological findings are showed in Supplementary information.

4.3.2. IMMUNOPHENOTYPE.

Flow cytometry was performed on the nineteen dogs with suspected hematopoietic neoplasia. The remaining ten dogs with lymphocytosis composed of a mixture of lymphocytes were not submitted to immunophenotyping. The cases were grouped according to the immunological diagnosis.

4.3.2.1. T-ALL: Acute Lymphocytic Leukemia of T-cell origin.

A T immunophenotype was assigned to seven cases of acute (expressing CD34) leukaemias (case no. 4, 5, 6, 8, 12, 19, 22). The lineage was defined given the positivity either to common lymphocyte markers (CD5) or due to CD8 or CD11d positivity. The final diagnosis was achievable in four cases (case no. 4, 6, 8, 19) expressing CD8, even if at low percentages (20-28%); This is a marker of T cytotoxic lymphocytes. Each of the four cases expressed a peculiar pattern of surface antibodies: case no. 4, co-expressed high levels (77%) of CD11d; case no. 6, co-expressed MHCII in 50% of the cells; in case no. 8 the diagnosis of a T-ALL was supported by the co-expression of CD8 in almost half of the cells gated for CD5; finally case no. 19, didn't express any other marker than a low level of CD8. In the remaining cases, diagnosis of T-ALL was based on positivity to CD11d (cases no. 5, 12, 22), which is reported to be a marker both for T lymphocytes and monocytes.

One case (no.14) was diagnosed as a Large Granular lymphocytes (LGL) leukaemia: the pattern of expression showed positivity to CD4 and CD11d. The diagnosis was supported by the cytological finding of magenta granules in the cytoplasm of the cells examined. This type of leukaemia seems to arise from either cytotoxic T- cells or NK cells.

4.3.2.2. AUL: Acute Undifferentiated Leukaemia.

In four cases (cases no. 1, 7, 10, 16) a definitive diagnosis was not achievable by immunophenotyping. The determination of acute leukaemia was made according to the elevated expression of CD34 in all the cases, but further characterisation of the cells was not achievable due to lack of expression of any other lineage marker. These cases, however, expressed homogeneously pan-leukocyte markers such as CD18 and CD45. Moreover, only one case (case no. 16) showed high (>50%) positivity levels of CD90, generally considered an early T-cell marker or a clonally expanding cells marker. Unfortunately, the significance of CD90 expression in

hematopoietic malignancies in dogs is still unclear. In one case (case no. 10) it was possible to gate and characterise a separate population from the neoplastic one: these cells expressed CD4, Anti-Neut antibody and MPO, markers of mature neutrophils, which might represent a response to the concurrent neoplasia. Even more, interestingly, this dog did not show any sign of neutrophilia on the CBC.

4.3.2.3. B-ALL: Acute Lymphocytic Leukemia of B-cell origin.

Diagnosis of acute leukaemia (expressing CD34) arising from B lymphocytes was made in three cases (cases no. 2, 9, 20). The diagnosis relied on the expression of intracellular CD79a, namely in case no. 2 (20%), case no. 9 (36.84%) and case no. 20 (47%). Moreover, in the three instances expression of CD90 was conserved and high (median expression around 58%), while CD18 was expressed in one case (case no. 2, in the total cells gated). No positivity for CD45 was evident. No positivity to CD21 (considered a marker for mature B cells) was visible, supporting the early stage of the disease and the diagnosis of acute leukaemia.

4.3.2.4. Aberrant phenotype.

Two cases (case no. 17 and case no. 21) showed similar immunophenotype, with concurrent expression of B and T cell markers. Acute leukaemia was confirmed by positivity to CD34 and CD90, and these cells were recognised as leukocytes by the positivity of CD18 and CD45 (the latter lacking in case 21). Case no. 17 showed high expression of CD5 (around 50%) which is a marker for T lymphocytes. Case no. 21 showed a high (>50%) positivity to CD4, a marker for T-helper cells. Therefore, both dogs showed a slight positivity to the B lineage marker CD79a. Due to this presentation, the cell lineage could not be assessed, and a bi-phenotypic pattern was recognised.

4.3.2.5. T-CLL: Chronic Lymphocytic Leukemia of T-cell origin.

Case no. 13 was the only one diagnosed with chronic leukaemia due to lack of expression of CD34 and mature appearance of the lymphocytes on blood smear examination. In this case, the T phenotype was recognised by the clear expression of CD3 both extracellularly (90%) and intracellularly (76%), and CD5 (24%). The expression of MHCII also demonstrates the indolent nature of the disease.

4.3.2.6. AML- Acute myeloid leukaemia.

The diagnosis of leukaemia of myeloid lineage in case no. 11 (most likely myelomonocytic) was confirmed by the expression of monocytoid markers (CD14) and neutrophilic markers (CD4 and Anti-neut). Lack of expression of lymphoid markers also supported the final diagnosis. This type of leukaemia, and this case namely, generally expresses pan leukocytes markers such as CD18 (in 58% of the gated cells in this patient), CD45 (45%). Neoplasia was confirmed due to positivity to CD34 and CD90 (clonal expanding cell marker).

Results from flow cytometric assay are shown in **Table 11**.

Table 11. Results from FC analysis of the canine patients enrolled in the study. Results are shown as % of cells expressing the marker in the gated region.

Sample ID.	DIAGNOSIS	STEM CELL MARKER		PANLEUKOCYTE MARKER		T-cell markers					B cell markers		Miscellaneous			MYELOID MARKERS		
						T-cell			T-helper	cytotoxic	Mature						neut	
		CD34	CD90	CD18	CD45	CD3	cd3-12	CD5	CD4	CD8	CD21	CD79a	CD11d	MHCII	CD117	CD14	ANTI-neut	MPO
4	T-ALL	85		95						20			77%		79			
5		90	95	99	92								90					
6		57			32					35				24	50			
8		96						46		22								
12		85	70		83								77		91			
19		75	60	25	79					25								
22		54	76	98	75								35		27			
14	LGL	74	82	99					10				65					
1	AUL	94.9		98	84										81			
7		71		93														
10		21		61	28				48								19	55
16		64	56	21	45													
2	B-ALL	81.7	53	100	94							20			95			
9		72	82									37						
20		45	40									47		50				
17	BIPHENOTYPIC	74	69	33	78			44				22		26				
21		55	76	97					53			17						
11	AML	43	29	58	34				20					n		15	30	
13	T-CLL			98	48	95	76	20						50				

4.3.3. EXTRACTION DATA AND DNA ASSESSMENT.

Median DNA concentration was 47.8 ng/µL ranging from a maximum of 174ng/µL (case no.5, diagnosed with T-ALL) and a minimum of 9.7ng/µL (case no. 11 diagnosed with AML). In general, low contaminations by proteins and salt were achieved with median $A_{260/280}$ of 1.78 and median $A_{260/230}$ of 1.80.

In all the cases DNA integrity was demonstrated by the positive reaction to canine C-kit gene amplification by PCR. Only case no. 19 showed a weaker positivity on agarose gel, possibly due to the lower DNA concentration and purity values (concentration= 11.85 ng/µL; $A_{260/280}$= 1.75; $A_{260/230}$=1.2).

Concentration values and ratios are shown in **Table 12**.

Table 12. Extraction data and positivity to PCR amplification of the control gene C-kit for the canine patients enrolled in the study.

Sample ID	DNA concentration (ng/µL)	$A_{260/280}$ ratio	C-kit
1	138.5	1.84	pos
2	138.5	1.85	pos
4	120.35	1.81	pos
5	174.05	1.78	pos
6	12.95	1.6	pos
7	79.8	1.75	pos
8	128.7	1.83	pos
9	168.15	1.81	pos
10	157.3	1.86	pos
11	9.7	1.59	pos
12	52.45	1.7	pos
13	80.65	1.62	pos
14	64.85	1.65	pos
16	47.6	1.78	pos
17	10.2	1.05	pos
19	11.85	1.75	weak pos
20	52	1.68	pos
21	34.8	1.03	pos
22	15.5	1.6	pos
A	19.36	1.97	pos
B	68.93	1.62	pos
C	36.52	2.48	pos
D	43.68	1.73	pos
E	28.73	1.83	pos
F	66.78	1.83	pos
G	29.66	1.98	pos
H	16.5	2.16	pos
I	38.25	1.89	pos
J	36.61	1.94	pos

4.3.4. PARR RESULTS.

4.3.4.1. Rearrangement of TCRG locus.

Two different primer sets were used to detect clonal rearrangement for TCRG. The first, named TCRG, was expected to give clonal amplicons of approximately 111bp. The second primer (named STCRG), configured for a multiplex PCR, was supposed to provide multiple bands ranging from 80 to 205bp.

Of the analysed samples, 7 cases (case no. 1, 4, 8, 13, 14, 17, 21) gave clonal results for TCRG locus rearrangement, suggesting neoplasia of T cells. However, rearrangements of the locus were detected with higher sensitivity from the STCRG primer, whereas the TCRG gave a clonal band in two cases (case 8 and case 13) visible from a smeary appearance on the background, likely the polyclonal residual population of lymphocytes.

4.3.4.2. Rearrangement of IGH locus.

In two cases (case no. 9 and 12), the neoplastic cells revealed a clonal rearrangement for the IGH locus, both for FR2 and FR3, detected by two separate reactions (primers named IgH2 and IgH3 respectively). Prominent bands of approximately 250bp (IgH2) and 180bp (IgH3) were indicative of a clonal population of lymphocytes, likely of B lineage.

Clonality for both B and T cell rearrangements was detected in two patients (case no. 2 and case no. 6). Both dogs were positive for rearrangements targeted by IgH2 primer, and dog no. 2 rearranged clonally for IgH3 as well. Clonality for TCRG was detected in both dogs with the STCRG primer.

No clonality was detected for the primers used in 7 cases (case no 7, 10, 11, 16, 19, 20, 22) affected by neoplasia and in all the dogs affected by reactive lymphocytosis (cases A-J) (**Table 13**). The results from the reactions performed were consistent with a polyclonal population of lymphocytes, given the smeary appearance of the bands in the expected size area of the gel. In just one case (case no. 19), not specific products were visible, giving a negative result.

All the reactions were run in duplicate and carried out along with a negative, a clonal and a polyclonal control. Examples of gels from each primers are showed below (**Figure 12**).

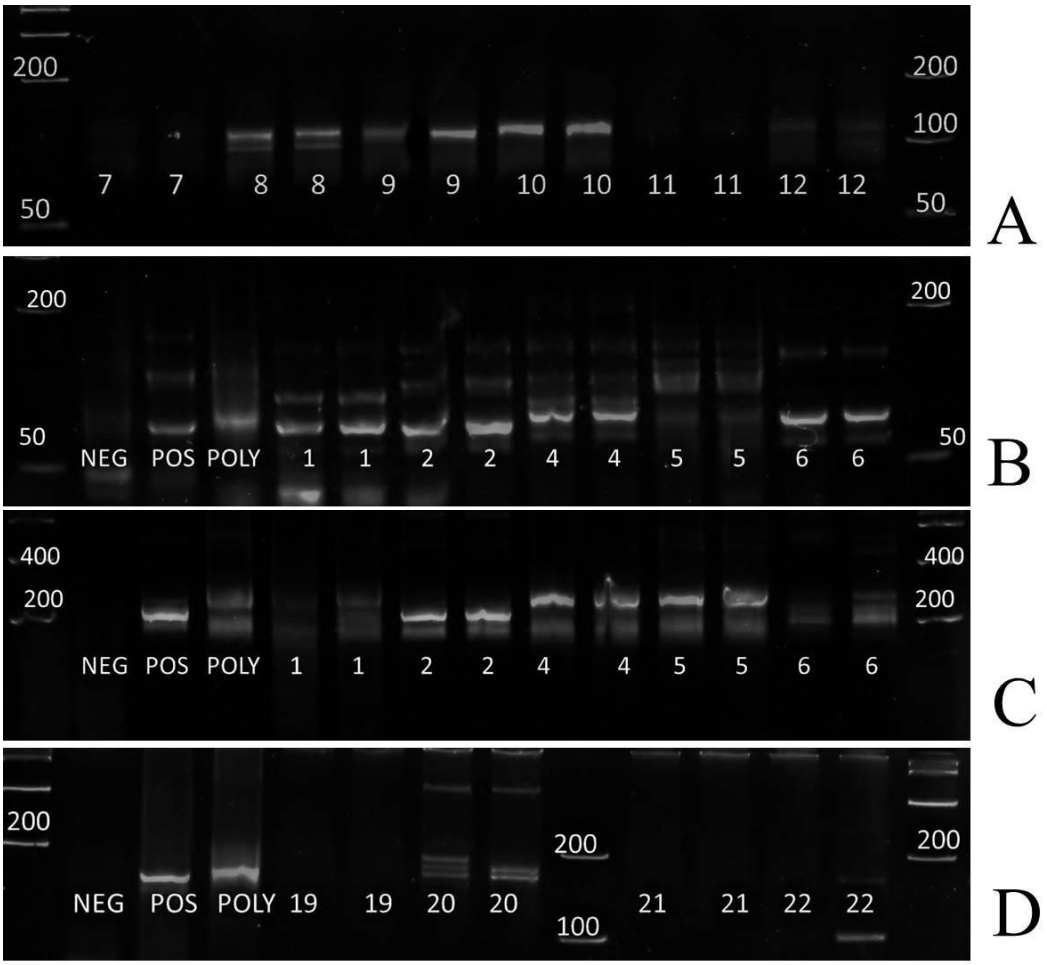


Figure 12. Examples of agarose gels for visualisations of PCR products.

Figure 12A, amplicons obtained from the reaction carried out with TCRG primer. Expected bands of approximate size 111bp. Sample no. 7 and 11, negative, no products visible. Sample 8, biclonal, with two bands visible in the expected area. Sample no. 9, pseudoclonal result; Sample 10, clonal, with the visualisation of a single band of expected size. Sample 12, polyclonal results, smeary band in the expected size area.

Figure 12B, amplicons obtained from the reaction carried out with primer named STCRG. Negative, positive and polyclonal controls are run along with the samples. Samples 1, 2, 4, 6, positive results, with visualisation of multiple bands ranging from 80 to 205bp. Sample 5, polyclonal result.

Figure 12C, amplicons obtained from the reaction carried out with primer named IgH2 (exp size of 250bp). Negative, positive and polyclonal controls are run along with the samples. Samples 2, 4, 5 monoclonal results, with visualisation of a single band measuring approximately 250bp. Sample 1 and 6, polyclonal.

Figure 12D, amplicons obtained from the reaction carried out with primer named IgH3 (exp size 180bp). Negative, positive and polyclonal results are run along with the samples. Samples 19, 21 and 22, negative for rearrangement; Sample 20, oligoclonal results, even though not exactly reproducible in the duplicates.

Size rulers are run along with the samples in all the gels. (4% Low melting point agarose, TBE buffer, 160V for 45 minutes).

4.3.5. FLOW CYTOMETRY VS PARR.

Four cases of T-cell neoplasia were confirmed on PARR. Rearrangements were detected using both primers in two cases (case no. 8 and 13), while STCRG primer was able to detect clonality where TCRG primer failed (case no. 4 and 14), increasing the sensitivity of the assay. Interestingly, case no. 14 was diagnosed by cytology and FC as having Large Granular Lymphocyte (LGL)-ALL.

One case of B-ALL was confirmed by PARR, by the visualisation of amplicons of the expected size on the gel, obtained with both IgH2 and IgH3 primers.

Cases of AUL and AML were also submitted to clonality assays: in particular two cases of AUL (case no. 7 and 10), and one case of AML (case no. 11) did not show any clonality when amplified with the primer set in use.

Immunophenotype and PARR disagreed on cell lineage in 11 cases (63% of the neoplastic cases, 41% of the total cases.

In most of the cases, the main reason for discrepancies in the final diagnosis was due to the failure of clonality detection. In three cases of T-ALL (case no.5, 19, 22) clonal rearrangement of TCRG locus was not detectable neither from Valli primer (TCRG) nor Keller primer (STCRG). B phenotype was missed in one case (case no. 20). In all these cases, however, immunophenotypic diagnosis relied on the expression of atypical patterns. Case 5 and case 22 for example, were diagnosed with T neoplasia, with the sole positivity to CD11d and CD90. No other lymphocytic markers were detected, except for the pan-leukocyte markers CD18 and CD45. The remaining T-ALL case (no. 22) of this group failed to yield clonality on PARR, and its immunophenotypic picture was also atypical: the cells were CD18+ and CD45+ (25% and 79% respectively) and CD90+ (60%); the cell lineage was assigned according to a low expression (25%) of CD8. No other evidence supported the final diagnosis. Failure in detection of IGH rearrangement was observed in case no. 20 which was polyclonal for both the reactions in use in this study: on FC the high positivity to CD79a (47%) was more convincing for B cell leukaemia, making the cause of failure in clonality detection to be based somewhere else, possibly in a mutated Ig locus.

In two cases clonal rearrangement of both IGH and TCR loci was detected: interestingly the two cases no. 2 and no. 6 were diagnosed by FC as having B-ALL

and T-ALL. In both cases, however, the final diagnosis was supported by a low percentage of only one marker: despite the high positivity in both cases to pan-leukocyte markers (CD18 and CD45), CD79a was positive in 20% of the cells in case 2 and CD 8 in the 35% of the cells in case 6. Moreover, in both cases, CD 117 was expressed. Case 6, also showed MHCII in 28% of the cells gated.

In two cases the diagnosis of AUL obtained by FC was refined by PARR, which gave clonal results for the primer STCRG, making the determination of T cell neoplasia likely. These two cases (no. 1 and no. 16) were defined as AUL given the lack of expression of any myeloid or lymphoid marker, although maintaining the expression of the pan-leukocyte markers CD18 and CD45 in a high percentage of cells, supporting the suspicion of a malignant hematopoietic process.

Two cases (sample no. 17 and 21) clonal for TCRG locus rearrangements (both retrieved by the use of the STCRG primer, and one clonal by TCRG as well), could be diagnosed with only PARR as T-ALL: interestingly the result of flow-cytometry showed a double phenotype. Case no. 17 was CD5+ in 44% of the cells, but low positivity (22%) to CD79a was evident, and the same pattern was shown by the case 21, with 53% of cells CD4+ and a small percentage (17%) CD79a.

One case (no. 12) was diagnosed with T-ALL by FC, due to the expression of CD18, CD45 and CD11d considered as a marker of T cells: PARR results gave instead clonal results using both primers targeting Ig locus (IgH2 and IgH3), typical for B cell neoplasia.

Results are schematically illustrated in **Table 13**.

Table 13. PARR results and final comparison to immunophenotype obtained by FC. Data about positivity to the PCR of the control gene C-kit are also shown. The green label is indicative of agreement between the two techniques. Conversely, the red label indicates disagreement. **PC**, polyclonal; **C**, clonal; **N**, negative; **PS**, pseudoclonal.

	C-kit	TCRG rearrangement		IgH rearrangement		COMPARISON		
		TCRG	STCRG	IgH2	IgH3	FLOW		PARR
4	Pos	PC	C		PC	T-ALL		T-ALL
8	Pos	C	C		PC	T-ALL		T-ALL
13	Pos	C	C	PC	N	T-CLL		T-ALL
14	Pos	PC	C		PC	LGL-ALL		T-ALL
9	Pos	PC			C	B-ALL		B-ALL
7	Pos	PC		PC	N	AUL		Non-clonal
10	Pos	PC			PC	AUL		Non-clonal
11	Pos	PC			PC	AML		Non-clonal
5	Pos	PC			PC	T-ALL		Non-clonal
19	weak pos	N			N	T-ALL		Non-clonal
22	Pos	PC			PC	T-ALL		Non-clonal
20	Pos	PC			PC	B-ALL		Non-clonal
2	Pos	PC	C		C	B-ALL		both rearr
6	Pos	PC	C	C	PC	T-ALL		both rearr
1	Pos	PC	C		PC	AUL		T-ALL
16	Pos	N	C	PS	N	AUL		T-ALL
17	Pos	PC	C	PC	PS	biphenotypic		T-ALL
21	Pos	PC	C	PC	N	biphenotypic		T-ALL
12	Pos	PC			C	T-ALL		B-ALL
A	Pos	PC			PC	R		R
B	Pos	PC			PC	R		R
C	Pos	PC			PC	R		R
D	Pos	PC			PC	R		R
E	Pos	PC			PC	R		R
F	Pos	PC			PC	R		R
G	Pos	PC			PC	R		R
H	Pos	PC			PC	R		R
I	Pos	PC			PC	R		R
J	weak pos	PC			PC	R		R

To summarise:

Of the twenty-nine cases included in the study, PARR detected neoplasia in twelve of the true nineteen neoplastic cases (63%), while all the reactive processes were recognised as polyclonal by the assay.

PARR accounted for a diagnostic sensitivity of 73% and a specificity of 100%, as none of the reactive processes was misdiagnosed as leukaemia. In fact, fewer false positives were detected by PARR when compared to true positive results, giving a predictive value of a positive test of 100%; moreover, the predictive value of the negative test was around 60%, showing that PARR has a medium probability of diagnosing a true negative as negative. Finally, the accuracy of the analysis was approximately 80%.

T phenotype was detected in 4 out of nine cases of T cell neoplasia. B phenotype was detected in one case of B cell neoplasia out of three.

Acute undifferentiated leukaemias accounted for a total of four cases: in half of them, PARR was able to retrieve clonality for TCRG locus, refining the diagnosis of leukaemia.

PARR also refined Biphenotypic leukaemia diagnosis: in both cases present in this study, the phenotype was clarified by PARR, revealing the rearrangement of the TCRG locus, compatible with a final diagnosis of T cell neoplasia. This result can also demonstrate how atypical expression of CD molecules can be present in neoplasia.

Finally, the only case of AML included was polyclonal for the rearrangement investigated: unfortunately, having a single case of myeloid neoplasia is not statistically analyzable for a more in-depth understanding of the clonality in the course of this disease.

4.4. DISCUSSION.

The present study investigates the ability of PARR to detect lymphoid neoplasia and refine the diagnosis, giving information about the clonality and phenotype of the cells examined. This is of some importance, as aforementioned for prognosis, as the cell lineage influences the biological behaviour and aggressiveness of the disease.

The assay failed to detect neoplasia in seven cases (36.8%). This high percentage of failure is however in accordance with previous reports. The causes have to involve both technical and biological pitfalls.

Technically, false negative results can be caused by the low amount of target DNA in the sample. This can be due either to the failure of the DNA extraction process or to the presence of few neoplastic cells in the samples. For this reasons, DNA-quality, controlled DNA-input and tissue representativity are essential to ascertain reproducible results.

Degradation of DNA can impair successful amplification by PCR. Old samples or FFPE tissues can have high levels of DNA fragmentation and degradation; although freezing/thawing is rarely a cause of DNA degradation in blood samples, DNA yield can be affected [140]. Moreover, the presence of PCR inhibiting factors (such as heme, lactoferrin, haemoglobin) could impair successful nucleic acids extraction and PCR[141]. In our study, we used frozen EDTA whole blood samples, which were thawed at RT; thus, the presence of PCR inhibitors and degradation of DNA cannot be excluded. Therefore, the DNA quality was checked, as recommended[142], on a control PCR analysis. Amplifying the canine C-kit gene resulting in positive in all the samples but one. In this case (case no. 19, T-ALL) the PARR amplifications for all the primers tested failed, despite the diagnosis of neoplasia given by FC. The low overall DNA quality and the negative amplification of the control gene could be a potential explanation for such results: highlighting that if on the one hand, PARR could be performed on virtually any source material, on the other an accurate upfront evaluation of extracted DNA is mandatory, and cannot be excluded from routine diagnostic tests.

False negative results can also be caused by too low an amount of target DNA in the sample, due to a low number of neoplastic lymphoid cells. In our study, however, we standardise the number of cells under examination to $9x10^6$/L with the aim of

eliminating a variable which could affect our trial. However, the final DNA concentration obtained was variable amongst the samples, and some of the yields were <20ng/µL with a lower $A_{260/280}$ ratio.

The low cellularity of a sample could potentially lead to the opposite result: if only a few amplifiable targets are present, even a polyclonal population can mimic a clonal one showing patterns that might look clonal. These results are defined as "pseudoclonal" and can be prevented by running the samples in duplicate or triplicate. Many studies recommend running each reaction in duplicates[27,58], as a true clonal pattern will give the same result in both PCR reactions, and possible artefacts can be avoided. In our study, all the samples were run in duplicates, and when a pseudoclonal result was yielded, the reaction was repeated. In only one case, the result persisted as pseudoclonality (n.17, IgH3 reaction). It was a dog diagnosed with "biphenotypic" acute leukaemia, as an expression of CD5 was evident, and a little positivity to CD79a (20%) was present too. The amplification of the IGH gene performed with the primer IgH3 yielded a pseudoclonal result twice using the same DNA template; unfortunately, we could not perform a second DNA extraction due to insufficient EDTA blood available. The explanation for this results could be the presence of very few B-cells in the sample (corresponding to the 20% expressing CD79a), with amplification of a restricted polyclonal pattern for IGH gene, giving a clonal appearance in one of the duplicates. The recommendation in these cases is to perform a second independent DNA extraction and to repeat the analysis[142]. However, the accurate description and evaluation of both immunophenotype and cellular morphology to confirm or exclude the presence of a small residual population different from the neoplastic one is mandatory in all cases, for consistency of the results.

Other explanations for failure in clonality detection, could be impairment of the functionality of the primers in use. Primers can fail in detecting clonality for two main reasons: they can show incorrect sequences, or they can lack complete coverage of the gene of interest. The first is a purely technical event: operator dependent errors are always possible. The second instance is a possibility that can occur, especially in Veterinary species, where knowledge of the complete genome is still not comprehensive. In our case series, positive, negative and polyclonal controls were included as recommended[63,142], and all the samples were run in the same reaction. The eventuality of a failure of the primer in just one case, out of a set of

reactions, is thus unlikely. Positive, polyclonal and negative controls should always be run along with the samples to detect primers failure or contaminations. However, this foresight cannot avoid incomplete gene coverage in the neoplastic cases.

As Ig/TCR gene rearrangements occur sequentially in the earliest stages of lymphoid differentiation, they are present in almost all immature and mature lymphoid cells, making their detection a reliable criterion of diagnosis of lymphoproliferative diseases. Knowing the gene structure, and the frequency by which single segments undergo rearrangement is essential in order to design efficient primers and efficient strategies aimed at clonality detection with the highest sensitivity possible. However, incomplete gene coverage can be attributable to the random mutations that can occur throughout the antigen receptor genes, especially during the so-called "somatic hypermutation" (SHM) of IGH-V segments that usually occur in antigen-stimulated B cells undergoing maturation in the germinal centers[143]. This mechanism occurs to add more variability to the pool of B cell receptors, and mostly comprises single-nucleotide mutations, deletions and insertions[144]. If on the one hand, the assessment of SHM can give information about the stage of B-cell development of a given leukaemia or lymphoma[145], on the other hand, it raises the issue of incomplete gene coverage. For this reason, in Human Medicine, the BIOMED-2 Concerted Action tested vast combinations of primers, resulting in fourteen final Ig/TCR multiplex PCR tubes. For B cell clonality, not only the heavy chain gene (including somatically mutated rearrangements) is targeted, but also the κ and the λ light chain encoding loci (IGK and IGL respectively)[95].

Incomplete gene coverage by primers directed to TCR gene can occur. For this reason, the primer set established by the BIOMED-2 guidelines for clonality of T cell neoplasms includes sequences targeting TCRB, TCRG, TCRD genes, although the latter could be omitted due to its complexity of interpretation[95,144]. An algorithm has been published in order to decide how to proceed with performing clonality assays in human lymphoproliferative diseases[144]. Usually the single detection of clonal rearrangement of TCRB and TCRG is enough to diagnose neoplasia, and usually, the genes are simultaneously rearranged. Conversely, TCRD is usually run when there is evidence of γδ lymphocytes proliferation or when only TCRG is detected.

When compared to Human Medicine, it is evident how impoverished the primer set designed for dogs is. In Veterinary Medicine, in fact, the only regions targeted are the TCRG (encoding for the γ chain of the TCR) and the IGH (encoding for the Heavy chain of the Ig). Although efforts have been made to design primers which are as practical as possible, the lack of knowledge of the canine genome is still an obstacle to design and targeting new genes. A possible solution would be to have more primers available in cases of suspected neoplasia which do not show clonality[64,67,69,70,81,87], even if some of them are included in the latest publications[72]. However, the recent description of canine TCRA/TCRD locus and IGHL is promising[86], and new tests are to be expected in the near future[58]. In fact, to clarify inconclusive clonality results, improving gene coverage and avoiding V segment mutations, the development of a TRD multiplex assay and an assay targeting the kappa-deleting elements (KDE) for dogs, cats, and horses are in progress[58], promising an improvement in clonality assays for companion animals.

Unfortunately, the visualisation method of PCR products was performed on Agarose gel, which has lower sensitivity than the modern automated capillary electrophoresis (CE), preferred for PARR analysis.[27] The visualisation method could severely impair the evaluation of clonality patterns, as a visual interpretation of gels is a non-standardized subjective technique and suffers from the possible imprecise reading of the bands. The gold standard for the PARR assay is CE a high-resolution assay. Other visualisation techniques include PAGE analysis[35,67,79], and recently a study tested the sensitivity and specificity of a High Resolution Melting analysis (HRM) method. The latter method is a post-PCR technique, which allows real-time detection of the double-stranded amplicons bound to detectable fluorescent dyes. It is an automated, fast, cost-effective technique, which can be done on qPCR instruments and that can be used in the diagnostic routine. This technique has been used in Human Medicine[146–149], and tested in both canine[150,151] and feline lymphoma clonality assays[152], but did not show a significant improvement in visualisation of PCR products. At the moment, the recommendation is to use capillary electrophoresis instruments: they do not require post PCR preparation, they can process a high number of samples in a short time, and they can give high-resolution results, with discrimination of few base pair differences between the amplicons. Moreover, they can quantify the results, making the interpretation less operator

dependent and more objective. Thus, the experienced operator is needed for interpretation of the results, especially in complicated or ambiguous cases.

In the present study, neoplasia was successfully detected by PARR in twelve out of twenty-nine cases (41%). Moreover, all the cases showing a mixed population lymphocytosis were diagnosed as reactive by PARR.

In our case series, PARR detected TCRG locus rearrangement successfully in ten cases of confirmed neoplasia. IGH locus was detected as clonal in four cases: two cases of confirmed B cell neoplasia, and two cases of T-ALL. Two cases, in fact, showed double rearrangement of both loci.

The clonal rearrangement for the antigen receptor gene of T lymphocytes was investigated using two primers. The first primer's sequence, named TCRG conventionally, was first published by Valli et al. in 2006[79] and was reported to give amplicons of 111bp approximately. A forward primer directed to the V segment of the CDR3 of the TCRG locus was coupled with a reverse primer, placed into the J sequence of the same gene. In the original study, the use of this primer allowed the detection of T cell neoplasia in five out of the eight lymphomas positive for CD3 (with a sensitivity of 62.5%). Therefore, the second primer used, named STCRG, was designed by Keller et al. (2012)[72], based on the newly acquired knowledge of the rearrangement patterns of the TCRG locus in the course of T cell neoplasia[153]. Knowing which segments undergo rearrangement and with which frequency, allowed the design of a primer meant for multiplex PCR amplification: the combination of different primers annealing the V and J segments of different cassettes of the gene, improved gene coverage and quantitative sensitivity of the assay. In two T-cell neoplasms of our case series, STCRG was able to amplify the target gene, despite the failure of the TCRG primer.

In our study, clonality detection using STCRG primer resulted in positivity in ten out of nineteen neoplasias (52.5%): two T-ALL, one T-CLL and one LGL-ALL; two cases of biphenotypic leukaemia and two AULs; in two cases (one B-ALL and one T-ALL) the primer retrieved prominent bands, but contemporary rearrangement of IGH was detected. On the other hand, the TCRG primer was able to detect neoplasia in only two of the total nineteen leukaemias (10.5%), one T-ALL and one T-CLL with an overall sensitivity of 72.5%. These results are in agreement with the original studies for TCRG, while overall sensitivity is slightly lower for STCRG primer [72,79],

which seems to be able to detect more rearrangements than TCRG primer. However, our case series included a varied group of diseases, such as B cell neoplasia, myeloid and undifferentiated leukaemias, thus making a true comparison of the efficiency of the two primers challenging. Therefore, STRCG primer, as aforementioned, allows the simultaneous detection of different rearrangements within the alleles or different neoplastic clones within same cancer: this is visualised as multiple peaks on CE, and multiple bands on gels.

Nevertheless, in the original study, the test resulted in a difficult interpretation for many reasons. Firstly, the presence of multiple bands makes the individuation of a real clonal population placed within a polyclonal background of reactive lymphocytes challenging. Moreover, T cell lymphoma showing different clones or different rearrangements per allele can be difficult to distinguish from reactive hyperplasia with a marked reduced TCR repertoire, as they can both result in multiple bands/peaks. All this together makes the STCRG a sensitive test for detection of neoplasia but complicates the recognition of true polyclonal cases. It was also true in our case series and especially on an agarose gel.

In the cases of confirmed T-cell neoplasia, we can be confident of our PARR diagnosis: clonality was detected in a lesion which was firstly evaluated by cytology, then characterised by FC. Whereas the pan leukocyte markers were all clearly expressed, the cell lineage was identified by a weak (20%) expression of the cytotoxic T cell marker CD8. These cases could enhance the possible role of PARR in refining the FC diagnosis, especially in cases with low marker expression rates. It is possible that due to the early maturation stage of the disease (confirmed by positivity to CD34), the mature marker expression was beginning to decrease; in this case, as the TCRG locus is the first rearranged and then maintained throughout all the lymphocyte maturation stages[154,155], its amplification could give information about cell lineage, even in blastic diseases. However, the low number of cases in this study allows just suppositions. More extensive studies are thus needed to evaluate the sensitivity of PARR in detecting immunophenotype in early blastic leukaemias, were immunophenotype is stiil controversial.

One case of LGL-ALL was included in the case series: the most prevalent markers expressed (apart from CD18, the pan-leucocyte marker) were CD11d and CD90, which are expected with large granular lymphocytes (NK cells or cytotoxic T

lymphocytes). The diagnosis was also supported by the microscopic visualisation of magenta granules within the cytoplasm of the circulating lymphocytes. Acute Leukaemia of LGLs is an aggressive disease, and most of the dogs die or are euthanised within a short time from the diagnosis[39,138]. LGL leukaemias can arise both from T-cells (expressing the CD3/TCR complex) or from Natural Killer (NK) cells; in both of cases, they rearrange TCRG genes[156]. In our case, due to the negativity to CD3, we suspect neoplasia of NK cells, and TCRG rearrangement was detected by PARR, confirming the phenotype and the cell lineage.

Two cases (case no. 17 and case no.21) showed double positivity to T-cell and B-cell markers on FC. Case no. 17 showed (apart from pan-leukocyte markers) positivity to CD5 and low positivity (22%) to CD79a. Case no. 21, similarly, showed positivity to CD4 and low positivity to CD79a (17%). Both expressed CD90 in high percentages (79% and 76% respectively), which is reported to be a T cell marker[38]. The clonality assay detected a clonal population of lymphocytes rearranging TCRG locus, using the primer STCRG alone. The cell lineage, refined by PARR, allowed the diagnosis in both cases of T cell neoplasia. This result highlights the variable significance that low levels of marker expression in FC can have: in Human Medicine, a cut-off of 20% expression was established in order to report as "positive" a given neoplasia for a given marker, and lower percentages should be reported as "negative"[157], with some exceptions[158]. In Veterinary Medicine there is still no consensus, but the use of positive controls or isotype controls (as performed in this study) is encouraged to define the background staining and autofluorescence correctly. As in Human leukaemias, the threshold of 20% was suggested to define positivity[159]. It is possible that in these cases, the lower percentage of cells showing positivity to B cell markers should have been reported as not significant.

In two cases, diagnosis of AUL, a rare ambiguous disease in dogs, was made and clonality of TCRG gene was detected. In previously published studies, the criteria by which the diagnosis of leukaemia as "undifferentiated" was made was not standardised, making possible the misdiagnosis of AML as AUL. In one study[160], in fact, AMLs were diagnosed based on the sole lack of expression of CD3 and CD79a. In other studies[47,161] and the present one, AULs were defined if lack of expression of lymphocytic or myeloid markers was evident. It is true, however, that the antibody panel and the criteria for the diagnosis of myeloid leukaemias were not very

comprehensive in any of the studies cited, neither in ours. Stokol et al.[38] have proposed an algorithm for AML diagnosis, which considers a wider antibody panel and ALP cytochemical staining as a useful tools in the diagnosis of AML[135]. Given these considerations, it is possible that some true AMLs have been misdiagnosed as AULs in our study. Moreover, we could speculate that, given the possibility of canine AML having clonal rearrangements for B or T cells[38], it could be possible that the two cases (no. 1 and no. 16) in this study, could eventually be of myeloid lineage. However, not all the AML cases clonally rearrange for TCR or IgH genes. The final diagnosis of AML should rely more on immunophenotypical or cytogenetic features (the latter adding prognostic information in Human Medicine[162,163]). It would be of interest to perform large-scale studies, integrating all this information and also investigating survival times and course of the disease. It has been shown in fact that there is a more aggressive behaviour of AML when compared to AULs, with poorer prognosis (personal data, unpublished).

In all the cases where clonality retrieved by STCRG primer and flow-cytometry did not agree, the interpretation of the clonality assay can be controversial. This primer, in fact, despite enhancing the qualitative sensitivity (ability to recognise a purely clonal population), showed inferior quantitative sensitivity (or ability to detect a clonal population within a reactive polyclonal one) in the original study[72], when compared to others[39,64,68,71]. The possibility of recognising a reactive population with a restricted TCR repertoire would be virtually impossible to distinguish from the presence of multiple malignant clones within the same neoplasm, especially on an agarose gel. We cannot, therefore, exclude the presence of a profound reactive reaction in the biphenotypic cases diagnosed by FC, in the AUL cases, or the cases were clonal rearrangements of both TCRG and IGH were detected, due to the limitation of the use of this primer.

The latter cases (case no. 2 and 6) are of specific interest. Detection of simultaneous rearrangements of IGH and TCRG are not uncommon in Human leukaemias and lymphomas[164,165] and seem to occur more often in B phenotype diseases[166,167]. This could be related to the reaction of T lymphocytes to the neoplasia: there can be depletion of T cell numbers and functions[168–174] or conversely, the evidence of oligoclonal populations in the peripheral blood, with an impairment of CD4+ lymphocytes and an increase in CD8+ cells[175], as has been demonstrated in CLL[176], multiple myeloma[177,178], and ALL[167,179,180].

In our case series, in case no. 2 (a B-ALL on FC) the rearrangement of IGH was detected by both primer sets, while only STCRG primer detected T cell clonality. Despite no evidence that residual or reactive T cells were present, we cannot claim that the neoplastic population showed contemporarly both rearrangements: T cell clonality in fact was detected by the STCRG primer, that, as already mentioned has a low analytical sensitivity. The presence of a small residual population of T cell could be evident on FC, but not in the gated population. This highlights the importance of the integration of the different techniques, from microscopic assessment, through immunophenotyping, and clonality assays. The second case (dog no. 6, diagnosed as having a T-ALL by FC, and showing rearrangement of IGH by primer IgH2), could be potentially true bi-rearranged neoplasia. Unfortunately, Agarose gel analysis allows just supposition, as it is a low sensitive visualisation technique. True lineage infidelity could be detected by single-cell analysis[181,182] which is a difficult technique that, to our knowledge, has never been applied in Veterinary Medicine. More sensitive separation techniques (such as CE or PAGE) are recommended in these cases.

Lack of optimal discrimination between a polyclonal and a clonal population can occur if when during the last PCR cycle steps, a high number of mispaired hemihelix are produced. This hemihelix, given the high sequence variability in the V-J junction, have a high probability of finding the homologous partner and to anneal to it.[139,183]. This way, distinguished between annealed heteroduplexes and homoduplex sequences could be challenging. To prevent this event, heteroduplex analysis can be performed, and it is highly reccomended[70,79,87,123]. After heat-driven denaturation of both homo and heteroduplex, a slow reannealing phase will be performed, allowing the perfect double-stranded homoduplexes to be produced, while the annealing of heteroduplex will not be perfect, as the sequences will differ in some part of the sequence. Heteroduplexes run on a gel will consequently result in a smeary band. In our study, we perform heteroduplex analysis, heating all the samples at 95°C for five minutes allowing denaturation of all the samples; consequently, we performed slow reannealing, lowering the temperature to 4°C and incubating the samples for one hour. We excluded, in this way, the possibility of a false positive result, believing that all the clonality yielded in the study was real.

Clonal results in the absence of neoplasia can occur in the case of the so-called “canonical rearrangement”, a process that determines a minimal diversified CDR3

due to minimal nucleotide additions during VJ junction[184–186]. In the dog, however, some studies have been carried out on the VJ usage of canine T cells[72], but the significance of some of the findings is still to be clarified.

These results highlight how PARR is not a technique meant to be run alone outside a complete diagnostic context. Clonality assays must not disregard microscopic evaluation and immunophenotyping in any case, and its usefulness is mostly appreciated in the distinction between reactive and neoplastic conditions, rather than immunophenotyping. In the case of negative or ambiguous results which are not consistent with the previous evaluation of the case, apart from the repeated molecular analysis, a careful combined (re)evaluation of the clinical, histopathologic, and molecular findings is needed to interpret oligo-/monoclonality results correctly.

4.5. CONCLUSIONS.

In conclusion, PARR is a powerful technique in the assessment of clonality in the course of lymphoproliferative diseases. Nevertheless, some precautions have to be taken when this assay is performed.

This assay can be performed on virtually any kind of sample: from FFPE tissue to fresh whole EDTA blood; from cell suspensions to cytologic and haematologic slides. However, DNA extraction is a crucial step for the final performance of PARR, as false negative results can be obtained in the case of poor quality DNA retrieval. Upfront evaluation of DNA is an essential step, which has to be regularly performed in a diagnostics routine.

To improve the sensitivity and the reliability of the assay, all the reactions have to be run in duplicate or triplicate; the event of pseudoclonal results is thus prevented by the detection of none reproducible results within the duplicates.

Primer sets have to be the most comprehensive possible, to avoid possible insufficient gene coverage: when neoplasia is strongly suspected, the use of different primers from the original routine primer sets, have to be included. In the case of primers designed for multiplex assays, the possibility of running singleplex assays using single combinations of forward and reverse primers have to be considered. This would potentially allow to distinguish between true clonal and polyclonal cell populations.

Automated detection techniques have to be preferred to the old gel electrophoresis analysis: such instruments assure greater accurancy, better resolution, and shorter processing times.

An interactive interpretation model, with regular contacts between clinicians, molecular biologists, pathologists, haematologists, and immunologists guarantees the integration of all the information. This model has to exist and to be effective in all the facilities offering PARR as a diagnostic tool. An accurate and most informative possible diagnosis and characterisation of haematopoietic neoplasia is nowadays possible and will benefit the Veterinary Oncologist and the Veterinary patient, above all.

CHAPTER 5:

FELINE CLONALITY ASSAY.

5.1. AIMS OF THE STUDY.

The primary aim of this retrospective study was to refine the diagnosis of lymphoma achieved by flow cytometry using PCR techniques (PARR).

PCR techniques will be improved by evaluation of PCR product amplicons by three visualisations by three different electrophoresis techniques: Agarose gel analysis, PAGE analysis and Capillary Electrophoresis

PARR will be applied to archived cytology samples from cats with fresh cytology samples previously analysed by Flow Cytometry for immunophenotyping.

PARR product will be analysed by CE to detect a clonal population within a reactive population, refining the results obtained by the other two electrophoresis techniques.

These aims are based on the following:

1. The importance of immunophenotype in lymphoma/ leukaemia diagnostics.
2. The advantages regarding cost and time, flow cytometry could represent a valid alternative to other immunophenotyping techniques in the assessment of phenotype in lymphoproliferative diseases in both dogs and cats.
3. The sensitivity of PARR in detecting a clonal population within a reactive lesion,
4. The advantage of PARR which can be performable on virtually any sample type,
5. The lack of knowledge of feline clonality assays and the lack of comparison of this method with techniques other than IHC,
6. The study will be specifically aimed at refining the methods for PARR for use with a variety of tissue sample from cats.

5.2. MATERIALS AND METHODS.

For PARR studies of lymphoma in cats PCR techniques will be applied to archived cytology samples from cats with a previously reported diagnosis of lymphoma/leukaemia or reactive lymphoid tissue based on FC of fresh cytology samples from FNA's of suspected tissues and blood smears. Because there is limited information available on PARR methods in cats the technique will be evaluated using optimization methods for DNA extraction, DNA preparation for archived cytology samples and for PCR product analysis by three different electrophoresis techniques. Because there are limited numbers of monoclonal antibodies available for FC immunophenotyping in the cat compared to the dog , many samples are designated as lymphoproliferative /reactive rather than neoplasia. Therefore PARR product analysis using CE will be used in an attempt to detect clonal populations within mixed reactive populations of lymphocytes .

5.2.1. Case Selection.

The archive of the Clinical Pathology Laboratory of the Dick White Referrals (DWR) Veterinary Centre was interrogated, and all the feline samples submitted for immunophenotyping by Flow Cytometry from 2015 to 2017 were selected. Immunophenotyping was performed if microscopic assessment provided suspicion for a neoplastic process.

Cytological slides from these patients were then retrieved and selected according to the flow cytometric diagnosis: from a total of thirty-seven samples, we chose twenty cases with a definitive diagnosis of hematopoietic neoplasia (of B or T cell origin), fourteen cases with a diagnosis of a reactive process, given by a mixed population of lymphocytes and three cases with non-definitive diagnosis, where neoplasia could not be confirmed or ruled out. Another criterion for inclusion was the quality of the source material, as we intended to select cases which had more than one slide available and which had good cellularity microscopically. No restriction on the site of origin of the sample was applied.

Diagnosis of neoplasia was achieved if the aberrant expression of CD molecules on the surface of gated lymphocytes was detected. T lymphocyte malignancy was diagnosed if the expression of CD3, CD4, CD8 and CD5 was evident. An alternate expression of CD8 or CD4 was suggestive for T cytotoxic lymphocytes or T helper

lymphocytes respectively. Also, co-expression of these two markers was considered aberrant and consistent with a neoplastic process. B lineage was assessed if CD21 was expressed in the absence of any T cell markers.

Diagnosis of a reactive process relied on the identification of a mixed population of lymphocytes, expressing both T and B cell markers.

5.2.1.b Flow Cytometery Immunophenotyping.

Samples for FC were collected by FNA of lymphoid tissues or venepuncture from the jugular vein. The latter were placed into EDTA collection tubes, while the cells obtained by aspiration were placed into transport medium composed of RPMI (tissue culture medium) supplemented with 10% BSA (Bovine Serum Albumin) for preservation of the cells during transport to the testing laboratory.

The sample was mixed and an aliquote removed for determination of the total cell count (WBC on an Advia Haematology analyser). An aliquot of 50µL or 100µL was used for preparation of a concentrated cells smear (Cytospin followed by staining). The blood was smeared on a clean slide to allow microscopical assessment. The slides were examined by a Clinical Pathologist to determine if the cells were lymphocytes in good condition and should be submitted to Flow Cytometry.

FC was set up in the same way as described for the dog with a limited panel of antibodies used. These included the following as a standard cat lymphoma panel. Extracellular binding mAbs: CD 18 as a leucocyte common antigen as no CD45 which binds to cat lymphocytes is commercially available; CD 4, CD8, CD5, which label T lymphocytes; CD21 which labels mature B lymphocytes. Intracellular binding mAbs included in the panel are CD3 for T lymphocytes and CD79a which binds B lymphocytes and MPO for granulocytes. Labelled cells were analysed in the same way as dog cells.

5.2.2. Clonality Assay.

5.2.2.1. DNA Extraction.

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen)[112].

As the manufacturer does not provide a protocol for cytological slides, we optimise a method to ensure extraction of sufficient amounts of good quality gDNA, adapting other protocols[113,114] as shown in Chapter 2.

Each slide was cleaned and any oil residues removed by immersion in Sub-X clearing medium (Leica) and wiped with a clean tissue. The dried material was then moistened using 20µL of the tissue lysis buffer (buffer ATL provided with the kit) and scraped off with a clean slide. The material obtained was placed into a 1.5 mL microcentrifuge tube, with a mixture of ATL buffer and proteinase K (Qiagen) in the proportions of 4:1 (80µL and 20µL respectively)[114]. If more than one slide was available from the same patient, each slide was moistened with 20µL of ATL, and the amount of buffer in the microcentrifuge tube was adjusted accordingly.

In order to avoid any contamination of the final gDNA with RNA, the mixture was incubated with 4µL of RNAase A stock solution (Qiagen, 100 mg/mL) for 2 minutes at RT.

Lysis was then continued by adding a second lysis buffer (AL buffer, provided with the kit) and incubating the mixture at 56°C for 5-7 hours[114]. Final lysis was then performed adding 200µL of pure (96-100%) ethanol to the mixture, now referred to as the "lysate".

The lysate was transferred to the QIAamp Mini spin column, provided with the kit. Centrifugation at 6000xg (8000rpm) for 1 minute ensures DNA binds to the net of the column. A double purification was performed by two consecutive washing steps (using washing buffers provided with the kit).

Genomic DNA was finally obtained by incubating the elution buffer (AE buffer, provided with the kit) for 5 minutes at room temperature in the column and spinning the sample at 6000xg (8000 rpm) for one minute. Double elution step[113] with 50µL was performed, for a final volume of 100µL.

DNA was stored at 4°C short term (1-2 weeks) or -20°C (1-2 months) until analysis.

5.2.2.2. DNA Quality Assessment.

Quantification of nucleic acid present in the eluate was performed with a Nanodrop 1000 (ThermoFisher Scientific) spectrophotometer. The technique provides the DNA concentration (expressed as ng/µL) and information about protein ($A_{260/280}$) and phenol or salt ($A_{260/230}$) contamination[105]. DNA showing values at $A_{260/280}$ around 1.8 and $A_{260/230}$ around 2.0 were considered of high purity.

A small amount of eluate (2µL) was pipetted directly onto the measurement pedestal. The sampling arm was then lowered, and the sample was maintained in place between the two optical fibres by the surface tension. The corresponding elution solution used was used as a blank before each analysis.

Polymerase chain reaction (PCR) was performed to amplify the feline Androgen Receptor (fAR) gene, as suggested by Mochizuki et al.[77]. The sequence of the primers used is the following:

forward primer, 5’ CAC AAT GCC GCT TAC GGG GAC CT 3’;
reverse primer, 5' AGG GGG TCA CAG ACC CTG ACT CG 3'.

PCR amplification was carried out using the TopTaq® Master Mix kit (Qiagen), according to the manufacturer’s instructions[118]. The protocol is briefly described below. A mixture of 12.5µL TopTaq Multiplex PCR Master Mix, 2.5µL of 10xCoral Load Dye, 1µl of 50x primer mix was prepared and loaded into PCR tubes, one for each reaction. A variable amount of eluate containing template DNA and RNase-free water was then added assuring an amount of DNA corresponding to 100-120ng/µL and a total reaction volume of 25µL.

After a first activation of the polymerase by heating the sample at 95°C for 5 minutes, forty cycles of denaturation (30 seconds at 95°C), annealing (90 seconds at 68°C) and extension (30 seconds at 72°C) were performed.

Agarose gel electrophoresis was performed to visualise the PCR products. GelStar stain (Lonza) was added to the 2% agarose Tris-Acetate EDTA (TAE) buffer mix during gel casting, at a 1x concentration. For quantification of the amplicons, DNA rulers were loaded into the external wells of the gel: FastRuler Ultra Low Range DNA Ladder and FastRuler Low Range DNA Ladder (ThermoFisher Scientific).

A total of 5µL of the sample was loaded in each well, and the samples were run at a voltage of 80V for 60 minutes. Gels were scanned using the Gel Doc™ XR+ (BioRad) scanner, and the excitation and emission were set at 493 nm and 527 nm respectively, according to the stain requirements[119].

The reaction was considered positive if a single discrete band weighting approximately 189 bp[77] was visible on the gel.

In conclusion, the combination of the information above (adequate concentration, $A_{260/280}$, $A_{260/230}$) and the visualisation of the band corresponding to the fAR gene, confirmed the suitability of the extracted DNA for further analysis.

5.2.2.3. PCR for Antigen Receptor Rearrangement (PARR).

Clonality testing was directed to the detection of rearrangement of genes encoding for antigen receptors, composed of four different regions: the Variable (V), the Joining (J) the constant (C) and the Diverse (D).

In the current study, primers directed to the V and J regions of TCRG and of the Heavy chain of the Immunoglobulin (IGHV and IGHJ respectively) were used as forward and reverse primers respectively.

Primers previously published[77,78,122] were purchased (Eurofins Genomics). The primers were mixed in order to obtain a final concentration of 100pmol/µL, and a 50x primer mix was used.

B cell clonality was assessed by the detection of rearrangement of the IGHV1 and IGHV3 as previously described[77]. A primer named V_1F_2 targeted the framework region 2 (FR2) of the gene IGHV1. Gene IGHV3 was investigated with two primers (named IgH2[122] and V3F4[77]) directed to FR2 and another two directed to framework region 3 (FR3) named IgH3[122] and V_3F_3[77]. Primers IgH2 and IgH3, designed by Werner et al.[122] were prepared in two tubes, each containing one forward primer and a combination of the three reverse primers (J1, J2, JD). Primer V_3F_3 was prepared in a single tube, containing the forward primer in conjunction with the five J reverse primers (JR1, JR2, JR3, JR4, JR5). Primers V_1F_2 and V_3F_4 were prepared for a multiplex reaction combining the two forward primers and the reverse primer mix. A tube containing the single V_3F_4 primer was also set up.

The use of one primer (TCRG)[78] detected T cell clonality. The five forward primers were placed on the V regions (namely V1-2, V3, V4 and V5) in conjunction with three reverse primers, located in the J region (J1, J2, J3).

Sequences used for amplification and expected band size are listed in **Table 6**.

A small subset of samples was additionally investigated for other rearrangements of the IGHV1 and IGHV3 genes. These samples have been diagnosed as "reactive" on FC, and after the standard set of primers was run, there was leftover DNA available. We decided to run additional PCR amplification in order to exclude all the possible IGH rearrangements whit the primers available (namely V_1F_1, V_3F_1, V_3F_2.) Additional primers used are shown in **Table 14**.

Table 14. Primers used for additional PCR amplification of the feline IGH gene.

		NAME	TARGET GENE	TARGET REGION	SEQUENCE	EXP SIZE	REF.
Feline IgH	Forward primers	V_1F_1	IgHV1	*FR1*	5' GCT GGT GCA GTC TGG GGC TG 3'	310-380 bp	Mochizuki 2011[77]
		V_3F_1	IgHV3	*FR1*	5' GGT GGA GTC TGG GGG AGA CCT G 3'	310-380 bp	
		V_3F_2			5' GGG GGT CCC TGA GAC TCA CCT G 3'	270-340 bp	
	Reverse primers	JR1	IgHJ		5' GCY STC ACC AGG RYT CCY BGGC-3		
		JR2			5' GCT GYG ACH MTD GTT CCA YGG CCC 3'		
		JR3			5' GCG RTG AYC WGG GTR YCY TGG C 3'		
		JR4			5' GCG GTG ACC AGG GTC CCG GGG CCC 3'		
		JR5			5' GCC GTC ACC AGG GTT CCG ACG CC 3'		

The amplification was performed in duplicate for all the samples tested.

The reactions were carried out using the Type-it ® Mutation Detect PCR kit, according to the manufacturer's instructions[117]. Briefly, A mixture of 18.25µL Type-it Multiplex PCR Master Mix, 3.25µL of Q solution, 3.25µL of 10xCoral Load Dye solution, and 1.5 µl of 50x primer mix was loaded in individual PCR tubes. A variable amount of eluate containing template DNA and RNase-free water was added, in volumes assuring 100-120 ng/µL of gDNA and a final reaction volume of 37.5µL per tube.

Cycling conditions were different amongst the primer sets.

Cycling protocol for the reactions set with primers from Werner et al.[122] was: the first activation of the Polymerase at 95°C for 5 minutes. Then, five cycles of denaturation (94°C for 30 seconds) and annealing (70°C for 90 seconds) followed by another 5 cycles of denaturation (94°C for 30 seconds) and denaturation (68°C for 90 seconds); finally, 35 cycles of denaturation (94°C for 30 seconds), annealing (65°C for 90 seconds) were followed by a final extension of 10 minutes at 68°C.

Cycling protocol for the reactions set with primers from Mochizuki[77,78] included: a first activation of the Polymerase at 95°C for 5 minutes was followed by forty cycles of denaturation (95°C for 30 seconds), annealing (68°C for 90 seconds) and elongation (72°C for 30 seconds). A final extension at 68°C for 10 minutes was then performed.

All the samples were submitted to heteroduplex analysis, with an additional denaturation step at 95°C for 5 minutes and a slow reannealing at 4 °C for one hour. This step was performed to increase the sensitivity and allowed the distinction between the real clonal homoduplex and the false clonal heteroduplex[123,139].

The reactions were performed using a T 100 Thermal Cycler (BioRad).

Positive (clonal) and negative controls were run along with the patients samples; Patient samples (as described in Chapter 3) and cell lines, kindly provided by Dr Hammer from the Immunology Department of The Veterinary University of Vienna, were used as clonal controls. Namely cell lines MS4 (B-cell line)[124] and FT-1 (T-cell line)[125] were used. Other samples described in the chapter "Establishment of positive controls" were used if cell line DNA was not available for analysis. DNA free water was used as a non-template negative control (NTC).

5.2.2.4. Visualisation of PCR products.

Three different electrophoretic techniques were used for the detection of the amplicons.

5.2.2.4.1. Agarose Electrophoresis.

Low Melting Point (LMP) Agarose powder (Sigma) was dissolved in TAE buffer at a concentration of 4%. The mixture was then heated in a microwave oven until complete dissolution. Staining was performed on pre-cast gels using GelStar (Lonza) at a final concentration of 1x: stain was added to the liquid agarose at a temperature of 65/75°C. The mixture was then gently poured into the electrophoresis platform and allow to solidify.

When ready, the gel was placed into the electrophoresis chamber filled with TAE buffer.

Five µL of PCR products were then loaded into each well of the gel. Electrophoresis was conducted at 200V for 5 minutes. Voltage was then lowered to 160V, and the run was considered completed after 45 minutes.

DNA rulers were loaded into the external wells of the gel: FastRuler Ultra Low Range DNA Ladder and FastRuler Low Range DNA Ladder (Thermo Fisher Scientific).

After runs were completed, gels were scanned using the Gel Doc™ XR+ (BioRad) scanner. Excitation and emission wavelength were set at 493nm and 527nm respectively, according to the stain requirements[119].

5.2.2.4.2 Polyacrylamide Gel Electrophoresis (PAGE).

Vertical Polyacrylamide gels (Novex™ TBE Gels, 20%, 15 well) were used as the second electrophoresis support.

The gels were provided in plastic cassettes and were stored at 4°C until used. After removing the cassette from the pouch and washing with deionised water, the tape on the bottom of the cassette and the plastic comb were gently removed. The wells were then rinsed with Tris-Borate EDTA (TBE) buffer.

The run was conducted using the XCell SureLock ™ Mini-Cell Electrophoresis Chamber (ThermoFisher Scientific), and the gels were placed individually on the bottom of the chamber, filled with TBE buffer.

Samples were prepared mixing 5µl of the PCR products with 2µL of 5x Novex™ Hi-Density TBE Sample Buffer (ThermoFisher Scientific) and deionised water according to the manufacturer's instructions.

A volume of 6µL of the mixture was loaded onto the gel.

DNA rulers (FastRuler ™ Ultra Low Range DNA Ladder and FastRuler™ Low Range DNA Ladder -Thermo Fisher Scientific) were also prepared as previously described and loaded onto the gel.

The run was conducted at a voltage of 180 for 90 minutes until the bromophenol blue contained in the sample buffer (dark blue colour) reached the bottom of the gel.

Staining was performed afterwards, using a 1x solution of GelStar (Lonza) in TBE buffer. After opening the cassette, the solution was gently poured onto the gel, which was subsequently incubated for 30 minutes in the dark.[119]

Finally, gels were scanned using the Gel Doc™ XR+ (BioRad) scanner. Excitation and emission wavelengths were set at 493 nm and 527 nm respectively, according to the stain requirements[119].

Visualisation of amplicons of the expected size (**Table 6**, **Table 14**) was suggestive of a clonal result. The presence of smeary bands or no visible bands in the expected area of the gel was supportive of a polyclonal or negative result. Pseudoclonal results were given by the visualisation of the expected sized band in only one of the duplicates.

5.2.2.4.3. Capillary Electrophoresis (CE).

To further characterise PCR products, separation of the fragments was performed by capillary electrophoresis using the QIAxcel Advanced system (Qiagen).

The system is a multi-capillary electrophoresis instrument which allows high throughput, fast and fully automated analysis of DNA fragments. In this study, the QIAxcel DNA High-Resolution Kit (Qiagen) was used, which is suitable for analysis of PCR products ranging between 15bp and 1kbp, with a resolution of 3-5 bp when >500 bp fragments are measured. It allows rapid analysis of the fragments (12 samples in 7 to 20 minutes) in a 96-wells setup.

The separation is performed in capillaries, part of a precast gel cartridge. The capillary is injected into the sample for a specific time (injection time), and when an electric field is started at a particular voltage, the fragments are automatically loaded into the capillary. As nucleic acids are negatively charged, they migrate to the cathode, with a speed which is inversely proportional to the size: low molecular weight fragments will travel faster into the capillary, whereas high molecular weight ones will be slower. The fluorescent signal emitted is then detected by a photomultiplier detector which converts the emission signal into electronic data. Finally, the QIAxcel ScreenGel software can process the data and display them as an electropherogram or gel image.

The QIAxcel DNA High-Resolution Kit contains: **1) QIAxcel DNA High-Resolution Cartridge** (with smart key); **2) QX Intensity Calibration Marker,** required to correct for natural intensity reading variations between each capillary in the cartridge**; 3) QX DNA Dilution Buffer**, to assure full immersion of the capillaries in the sample and to normalize sample concentration. It can be diluted with **4) QX DNA Size Marker** in a concentration of 5ng/µl. The marker gives a reference size, which allows the calculation of the size of the DNA to be analysed by a point-to-point calculation. The marker contains a range of fragments, within which the expected size of the analysed samples should fall (the fragments of the marker used in this study, ranged from 25bp to 500 bp), each with an approximate concentration of 0.87ng/µl, except for the 300bp fragment which had a concentration of 2.17ng/µL; **5) QX Separation Buffer**; **6) QX Wash Buffer,** used to wash the capillary tip and avoid cross-contamination between samples; **7) QX Mineral Oil,** added to buffers to avoid evaporation; **8) QX Alignment Marker,** ranging from15bp/1kb. This marker is intended to calibrate the migration time variation

across all channels. It contains two fragments of known size (in this case, 15 bp and 1kbp, a range containing the size expected for analysed samples) and runs along with the samples, with which it co-migrates.

The buffer tray of the QIAxel Advanced system comprises three buffer positions (Wash Park, WP; Wash inject- WI; Buffer- BUF), two markers positions (Marker 1-M1; Marker 2-M2), and the sample loading area with 12 rows and eight columns (for a total of 96 wells).

Before using the cartridge for the first time, intensity calibration was required. This step was performed by loading 15µl of QX Intensity Calibration Marker into each tube of a 12-Tube Strip with a drop of mineral oil. The strips are placed into the M2 position of the buffer tray. The calibration is performed via the QIAxcel ScreenGel software, and it is considered completed if the normalised area of the cartridge ranges between 0.004–0.006.

The cartridge was stored at 4°C, and allowed to stand at RT for 20 minutes before use.

Before loading the samples, the WP and WI positions of the buffer tray were loaded with 8mL QX Wash Buffer, and**,** whereas the BUF position was loaded with 18mL QX Separation Buffer; 2mL and 4mL of QX Mineral Oil were added to the positions respectively.

The alignment marker was then added to a 12-tube strip, and placed in the M2 position of the buffer tray, and run along with the samples.

Sample volumes were normalised to 50µL by loading variable volumes of the QX DNA Dilution Buffer and QX DNA Size Marker mixture, depending on the PCR reaction volumes.

The run was performed using the QIAxcel ScreenGel software v1.3 using the OL500 setting: injection time was set at 20 seconds and 8 kV. Separation was then performed at 5kV for 500 seconds.

DNA analysis was performed by the QIAxcel ScreenGel software v1.3 using an algorithm which considers three fundamental steps. The first step was the construction of the baseline, made by taking a horizontal straight line that starts from the average signal values at the beginning of the electropherogram. Then, areas in the electropherograms where there was a significant difference between the baseline and the row data were detected: these areas are named “clusters” and their detection is made using the so-called “Threshold”, an imaginary line parallel to the constructed baseline indicating the minimal height of a peak (in reference to the

constructed baseline) to be detected. A step called “iteration” then optimises the baseline construction. Then, cluster splitting was performed, and clusters with several peaks were divided into several clusters. Finally, the peaks are detected, by accounting for minimum distance and alignment marker threshold. The maximum data point in the cluster corresponds to the apex of the peak; start and end points of the peak were then determined.

Results were interpreted according to published guidelines[58,63]. Clonal results were considered if one or more reproducible peaks in the expected size range were detectable: this was considered indicative of an ongoing neoplastic process. The detection of a Gaussian curve (with or without minor reproducible peaks) was indicative of a polyclonal result, likely due to the reactive process. Pseudoclonality was given if no reproducible peaks were detectable in the duplicates. Finally, the absence of signal was indicative of no specific products detection, compatible with a low concentration of poor quality DNA or mutated target DNA.

5.3. RESULTS.

5.3.1. EXTRACTION DATA.

According to previous studies[27], a concentration >30 ng/μL was considered optimal for further analysis, with $A_{260/280}$ of around 1.8 and $A_{260/230}$ of around 2.

Of the 37 cases selected, extraction was successful in 12 samples (two coming from the same patient but different locations, cat no. 25), fulfilling the optimal requirements. However, we decided to include some of the samples (nine in total) which showed good quality characteristics, even with low concentrations of DNA.

Seventeen samples were excluded due to low quality DNA yield.

Complete extraction data and DNA purity are shown in **Table 4S** of Supplementary Information.

5.3.2. CASE DESCRIPTION.

The twenty-two cases recruited from DWR were divided into two groups: the first one included fourteen cats with lymphoid neoplasia (according to evidence of atypical cells in the peripheral blood and the organ affected); the second group included eight cats showing a mixed population of lymphocytes, not attributable to any neoplastic disease. This evidence was supported by the microscopic assessment of the cells and flow cytometry.

The fourteen cases confirmed as neoplasia on Flow Cytometry were distinguished as B or T cell neoplasia, according to the positivity of CD21 and CD5 respectively. Further characterisation of T cell neoplasia was possible due to the expression of CD4 and CD8. T cell lineage was recognised in seven cases (57.14%) of the cases. In three cases (case no. 3, 7, 27) small mature lymphocytes were detected in the blood, and a final diagnosis of Chronic Lymphoid Leukemia (T-CLL) was made. Three cases were FNAs from lymph nodes: abdominal lymph nodes (case 31, abdominal lymph node; case 4, site not specified). One of these cats also had a mass on the left kidney (cat no.15), and another cat had a mass reported to be on the "caudal dorsum" (cat no. 34). Six cases expressed the B cell marker CD21. Half of them showed the involvement of the GI and lymph nodes, with the presence of a mass in the colon (cat no.22), in the intestine (site not specified, cat no. 24) and involvement of the jejunal lymph node (cat no.5). Lymph nodes were involved in three cases (cat no.18, prescapular node; cat no 36, site not specified). In one case (cat no. 10) immunophenotyping was performed on blood. Interestingly, in all the cases, the cytology reported the presence of intermediate to large lymphocytes with features supporting a diagnosis of high-grade lymphoma (**Table 15**).

About signalment, all the cats of this group were DSH, except one DLH. The breed was unknown in one case. The median age was 8.8 years. No clinical information about the cases was available.

The confirmed reactive processes on flow cytometry were five in total. Immunophenotyping allowed separation of different populations expressing B and T cell markers, which were within physiologic percentages. All the cats were Domestic Short Hair (DSH) except one Domestic Long Hair (DLH) and one Burmilla. The breed was unknown in one case. The median age was 7,8 years. No clinical information was available for these cases (**Table 15**).

Most of the samples consisted of FNAs of lymph nodes: in four cases the location was not specified (case no. 12, 16, 17); one sample (no.32) came from the submandibular lymph node and one from an abdominal lymph node. One cat (no.25) was sampled in two different locations: mesenteric lymph node and spleen, which were infiltrated by the same lymphocyte population.

In three cases, the diagnosis given was defined as "highly suggestive of", as neoplasia or reactive hyperplasia could not be completely ruled out. In particular, case no. 28 showed a mixed population, but within the T cell population "dual staining with CD4 and CD8 showed a predominance of CD8 cells over CD4 cells", which could reflect a CD8+ T cell neoplasia or hyperplasia. In case no. 29 a mixed population of B and T cells was present, but a small percentage of cells co-expressed CD4 and CD8, which is aberrant. Due to the low percentage, however, a final diagnosis could not be made. Similarly, in case 33 "A high proportion of cells have stained with CD5 (T cell marker) with a low proportion of CD21 positive B cells (…). The majority of the T cells express CD4 and only a low proportion express CD8 (....).When gating the larger cells, the proportion of CD5+ CD4+ increases with an absence of B cells". This could be suggestive of T cell lymphoma, but profound reactive hyperplasia could not be ruled out. The twenty-two cases are described and summarised below in **Table 15**.

Table 15. Data about the twenty-two feline samples enrolled in the study. **N-T**, Neoplasia of T cell; **N-B**, neoplasia of B cells; **R**, reactive process; **ND,** not a definitive diagnosis; **LN**, lymphnode.

Sample ID	Immunophenotype	Age (years)	Breed	Sample type	N.of slides used	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
3	N-T	12	DSH	Blood	1	14	1.775	1.7
4		NA	NA	LN	1	33.4	1.81	1.8
7		13	DSH	Blood	2	14.36	1.88	1.6
15		14	DSH	Renal mass	2	74.43	1.82	1.86
27		13	DSH	Blood	2	22.95	1.78	1.5
31		NA	DSH	Abdominal LN	2	19.6	1.81	1.6
34		2	DSH	Mass on caudal dorsum	2	45.8	1.85	1.73
5	N-B	10	Birman	Jejunal LN	1	22.2	1.915	1.66
10		10	DSH	Blood	1	44.7	1.81	2.13
18		5	DSH	Prescapular LN	2	82.00	1.93	2.65
22		13	DSH	Colonic mass	2	22.3	1.88	1.39
24		12	DSH	Intestinal mass	1	12.1	1.94	2.19
36		11	DSH	LN	3	500	1.96	2.11
12	R	1	DSH	LN	3	8.1	1.97	1.73
16		11	DSH	LN	2	103.8	1.83	2.06
17		14	DLH	LN	3	119	1.87	2.22
25		8	DSH	Mesenteric LN	2	59.5	1.91	1.32
				Spleen	2	153.9	1.94	1.86
32		8	Burmilla	Submandibular LN	2	34.4	1.81	2.12
28	ND	NA	NA	Abdominal LN	3	21	1.9	1.73
29		5	DSH	LN	2	48.8	1.81	2.36
33		12	DLH	jejunal LN	2	140.4	1.87	2

5.3.3. PARR RESULTS.

5.3.3.1. Rearrangement of TCRG locus.

A single primer combination was used to detect clonal rearrangement for TCRG. The visualisation of a band of approximately 80-120 bp was suggestive of neoplasia[78].

5.3.3.1.1. Agarose gel electrophoresis.

On agarose gels, a prominent band in the size area representing a clonal result was visualised in five cases (case no. 3, 5, 7, 12, 15). In five cases (case no. 16, 17, 25 both locations, 33, 36) clonality was suspected, but the band visualised was not entirely convincing neat. (**Figure 13**).

No specific products were visible in cases no. 4 and 18.

Polyclonal results yielded smeary bands in the rest of the cases (10, 22, 24, 27, 28, 29, 31, 32, 34) suggesting detection of different rearrangements in a mixed lymphocytes population. All The results of TCRG gene amplification on Agarose gel are shown in **Figure 13**.

5.3.3.1.2. Polyacrylamide Gel Electrophoresis (PAGE).

Amplicons obtained from the TCRG reaction were separated more efficiently on PAGE, and the bands showed better resolution. Monoclonality was confirmed in case no. 3 and 5. Biclonal and oligoclonal results were distinguishable from pure monoclonal cases: namely, case no. 7 was assessed as "oligoclonal" (three bands visible) and sample no. 15 showed two distinct bands and was defined as "biclonal".(**Figure 14**) Interestingly, sample no. 29 assigned as "polyclonal" on agarose gave a clear band of the expected size, thus being diagnosed as "clonal". Sample no. 12, defined clonal on agarose, gave multiple bands, and the result was changed to "polyclonal". For the presence of multiple bands in the area of interest true polyclonal samples were clearer on PAGE: the presence of multiple bands distributed in the gel or a smeary appearance was evident in cases no. 10, 17, 22, 24, 25 (bot locations), 27, 28, 31, 33, 34. No visible bands were obtained from cats no. 4, 16, 18, 32, 36. Thus these were assigned as "negative".

The results of TCRG gene amplification on PAGE are shown in **Figure 14**.

5.3.3.1.3. Capillary Electrophoresis.

If PCR products were left from the previous analysis, capillary electrophoresis was performed using the QIAxcel Advanced system (Qiagen).

Clonality for TCRG locus was analysed in eighteen samples (no. 3, 4, 5, 7, 10, 12, 15, 16, 17, 22, 25 both locations, 29, 31, 32, 33, 34, 36). The results for samples no. 3, 7 and 15 mostly matched with the previous gel electrophoresis but had the advantage of giving a precise size for the peaks detected. Sample no. 3 gave a distinct peak of approximately 140bp. Sample no.7 was confirmed as oligoclonal, with the presence of multiple peaks (ranging from 129 to 154bp). Furthermore, sample no. 15 was confirmed as biclonal, with the presence of two distinct peaks of 140bp and 163bp. Despite the suspected monoclonality, sample no.5 gave on CE a polyclonal peak (**Figure 15**).

All the rest of the samples were polyclonal, given the visualisation of differently sized peaks, resembling a Gaussian-curve. Within the polyclonal- looking samples, sample 33 showed a more prominent peak, emerging from the polyclonal background. Suspecting a clonal result (also given the flow cytometric diagnosis), we carried out a singleplex reaction for TCRG primer, described below. Electropherograms from capillary electrophoresis are shown in **Figure 15**.

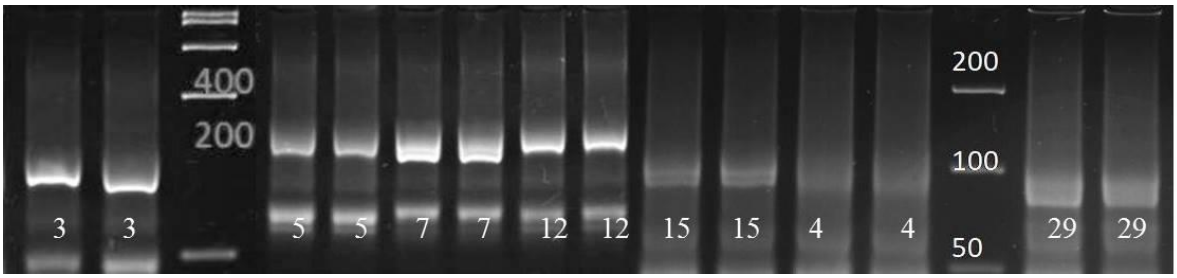


Figure 13 Agarose gel for visualisation of PCR product obtained from TCR primer amplification. **Sample 3**, interpreted as clonal; **sample 5,** interpreted as clonal; **sample 7,** interpreted as clonal; **sample 12,** interpreted as clonal; **sample 15,** interpreted as clonal; **sample 4,** interpreted as negative; **sample. 29,** interpreted as polyclonal. NTC and positive cell line FT-1 were run along with the samples (not shown).

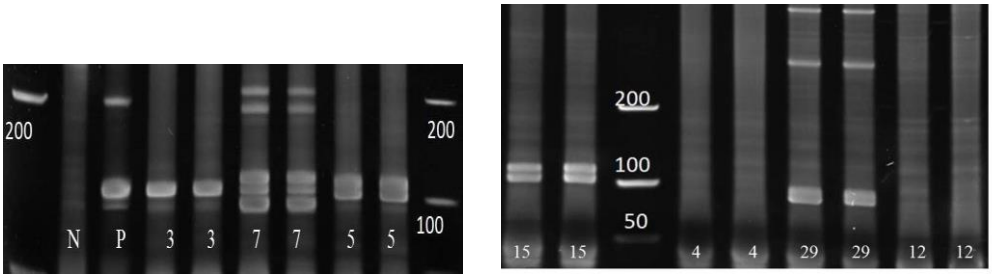


Figure 14. PAGE for visualisation of PCR product obtained from TCR primer amplification (expected size approximately 80-120bp). **Sample 3,** confirmed as monoclonal; **Sample 7,** interpreted as oligoclonal; **Sample 5,** interpreted as monoclonal **Sample 15,** interpreted as biclonal; **Sample no. 4,** confirmed as negative; **Sample no. 29,** interpreted as clonal; s**ample 12,** interpreted as polyclonal; NTC and positive cell line FT-1 were run along with the samples.

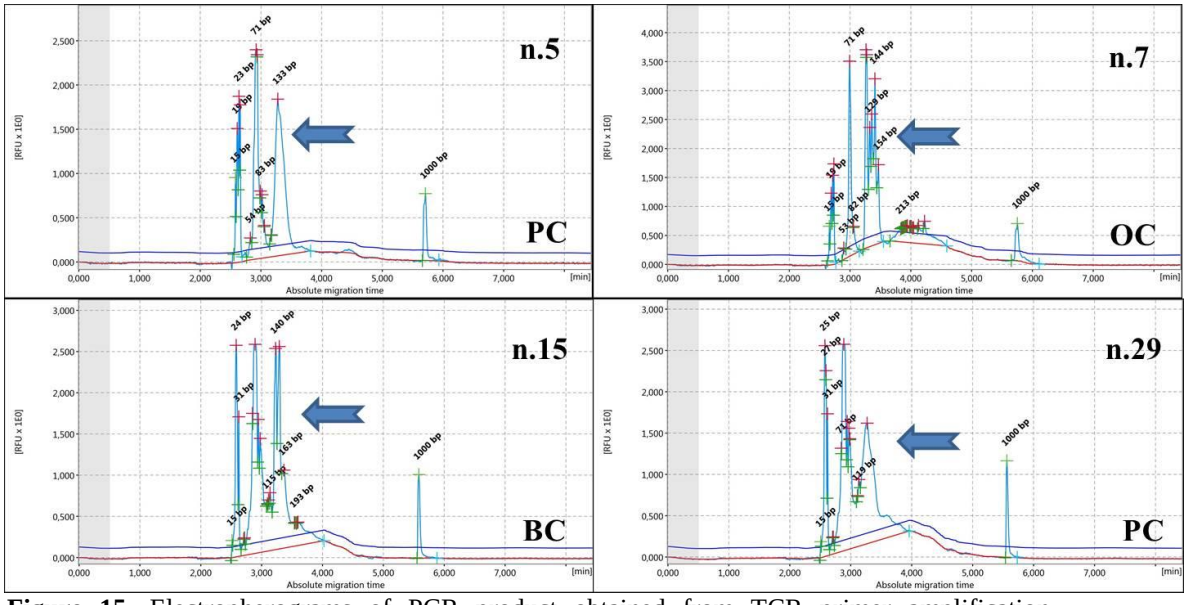


Figure 15. Electropherograms of PCR product obtained from TCR primer amplification (expected size approximately 80-120bp). **Sample 5,** interpreted as polyclonal **(PC)**; **Sample no. 7,** confirmed as oligoclonal **(OC)** for the presence of multiple peaks (of 129bp, 144bp, 154 bp-arrow); **sample no. 15,** confirmed as biclonal **(BC)** for the presence of two peaks of 140bp and 163 bp (arrow); **sample no. 29**, interpreted as a polyclonal **(PC)**. NTC and positive cell line FT-1 were run along with the samples (not. shown).

5.3.3.2. Rearrangement of IGH locus.

Rearrangement of the feline IGHV3 was investigated with four primers targeting FR2 (IgH2 and V_3F_3) and FR3 (IgH3 and V_3F_4) [77,122]. The FR3 region of the IGHV1 gene was investigated using the primer V_1F_2[77].

5.3.3.2.1. Agarose gel electrophoresis.

The primer IgH2 gave no clonal results on agarose gels.

The primer V_3F_3 was tested in all the samples but one (no.31), giving clonal results in sample 10 and 34. In sample no. 10, two bands were visible (in the area of the gel between 180-220bp). All the rest of the cats showed polyclonality, except for samples 3, 5, 7 and 15 which showed no products. Sample no. 22 was ambiguous showing a band in the expected size with a slightly blurry appearance. By agarose gel, the interpretation of this sample was challenging, and we defined it as "clonal". (**Figure 16A**).

The primer IgH3 was tested on 19 samples, giving monoclonal results in cat number 5 (**Figure 16C**). All the rest of the reactions were considered negative, except samples 4, 16 and 17 which show a smeary band and were interpreted as "polyclonal".

Finally, we tested the primer V_3F_4 in two different combinations: first, a multiplex reaction combining the V_3F_4 and the V_1F_2 forward primers with the J reverse primers was performed, in order to investigate these possible rearrangements by reducing the number of reactions targeting the FR3 of both IGHV1 and IGHV3. This reaction was tested in all the samples but one (case no. 3). The only clonal result was achieved for sample no. 10, with visualisation of a band of approximately 180 bp size on agarose gel. In two samples (no. 25 Lymph node and no. 34) a band was visible, thus not thoroughly convincing. Moreover, a singleplex reaction for V_3F_4 was performed on the 13 samples (cases no.4, 5, 12, 15, 16, 17, 18, 25 both locations, 29, 33, 34, 36) for which DNA was available, thus giving all negative results, except for samples 15 and 18, where a blurred area was visible. (**Figure 18**).

5.3.3.2.2. Polyacrylamide Gel Electrophoresis (PAGE).

The primer IgH2 gave no clonal results on PAGE gels.

Clonal amplicons obtained from V_3F_3 and IgH3 primers reactions were brightly visible on PAGE gels and confirmed the results obtained by agarose gel analysis. Only sample no. 22 that was diagnosed as "clonal" on an agarose gel showed a polyclonal pattern on PAGE gel. (**Figure 16B and 16D**).

Case no. 10 tested for the multiplex mix of V_3F_4 and V_1F_2, gave a bright clonal band on PAGE gel. Moreover, the band given by sample 34 and not as strongly prominent on agarose was clarified on PAGE gel, and the rearrangement was defined as monoclonal. Conversely, sample no.25 (lymph node) which appeared clonal on agarose, showed a polyclonal pattern on PAGE. All the singleplex reactions for V_3F_4 gave polyclonal or negative results. (**Figure 18**).

Figure 16. Agarose gel and PAGE scans for visualisation of feline IGH obtained by V_3F_3 and IgH3 primers.

Fig 16A Agarose gel showing a reaction with primer V_3F_3 (expected size 210-280bp). **Sample 3**, interpreted as negative; **Sample 4,** interpreted as polyclonal; S**ample 5,** interpreted as negative; **Sample 7,** interpreted as negative; S**ample 10,** interpreted as biclonal; **Sample 24,** interpreted as polyclonal; S**ample 22,** interpreted as clonal; **Sample. 32,** interpreted as polyclonal; **Sample. 33,** interpreted as polyclonal; **Sample. 34,** interpreted as monoclonal; **Sample. 36,** interpreted as polyclonal. NTC and positive cell line MS-4 were run along with the samples.

Fig 16B, PAGE showing a reaction with primer V_3F_3 (expected size 210-280bp). **Sample 3**, interpreted as negative; **Sample 4,** interpreted as negative; **Sample 5,** interpreted as negative; **Sample 7,** interpreted as negative; **Sample 10,** confirmed as biclonal; **Sample 22,** interpreted as polyclonal; **Sample 24,** interpreted as polyclonal; S**ample. 33,** interpreted as negative; **Sample. 34,** interpreted as monoclonal; **Sample. 36,** interpreted as negative. NTC and positive cell line MS-4 were run along with the samples.

Fig 16C, Agarose gel showing reactions with primer IgH3 (expected size 130-180bp). **Sample 3**, interpreted as negative; **sample 5,** interpreted as monoclonal;

Fig 16D, PAGE showing reactions with primer IgH3 (expected size 130-180bp). **Sample 3**, interpreted as negative; **sample 5,** interpreted as monoclonal; **sample 7,** interpreted as negative;

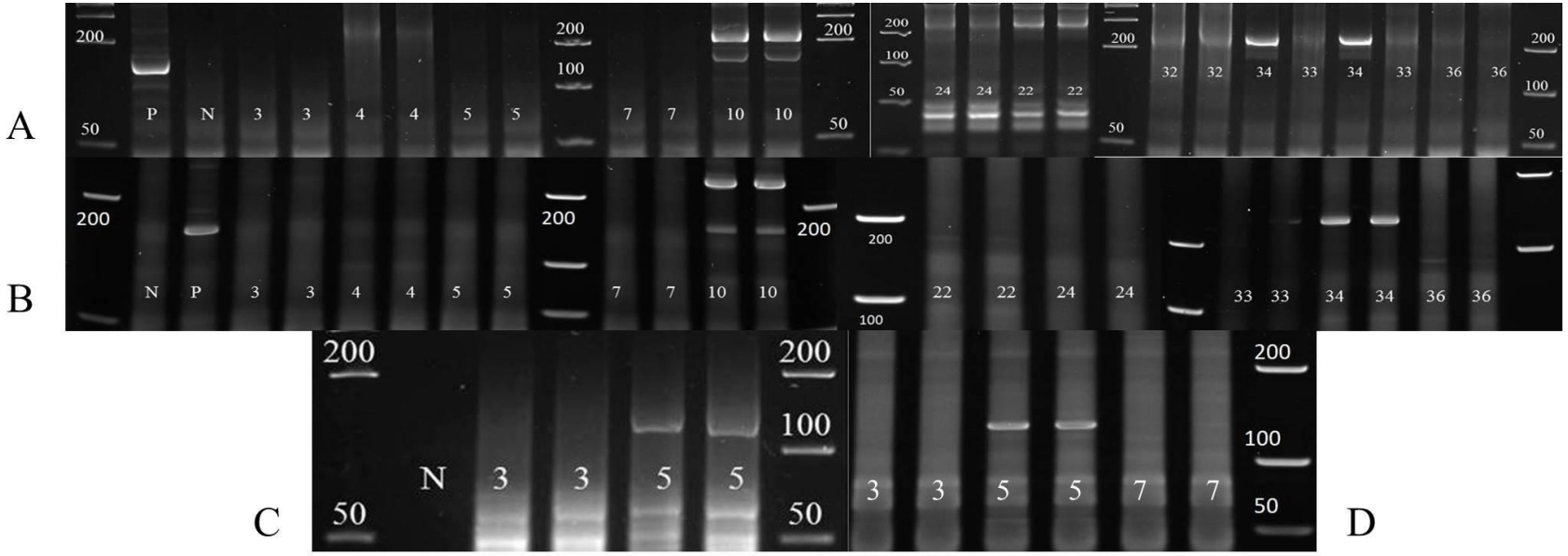


Figure 17. Electropherograms of PCR product obtained from V_3F_3 and IgH3 primers amplification.

Fig 17A, Sample 7, V_3F_3 reaction interpreted as negative **(N)**;**Fig 17B, Sample 10,** V_3F_3 reaction confirmed as monoclonal for the presence of a peak of 202 bp **(M)**;

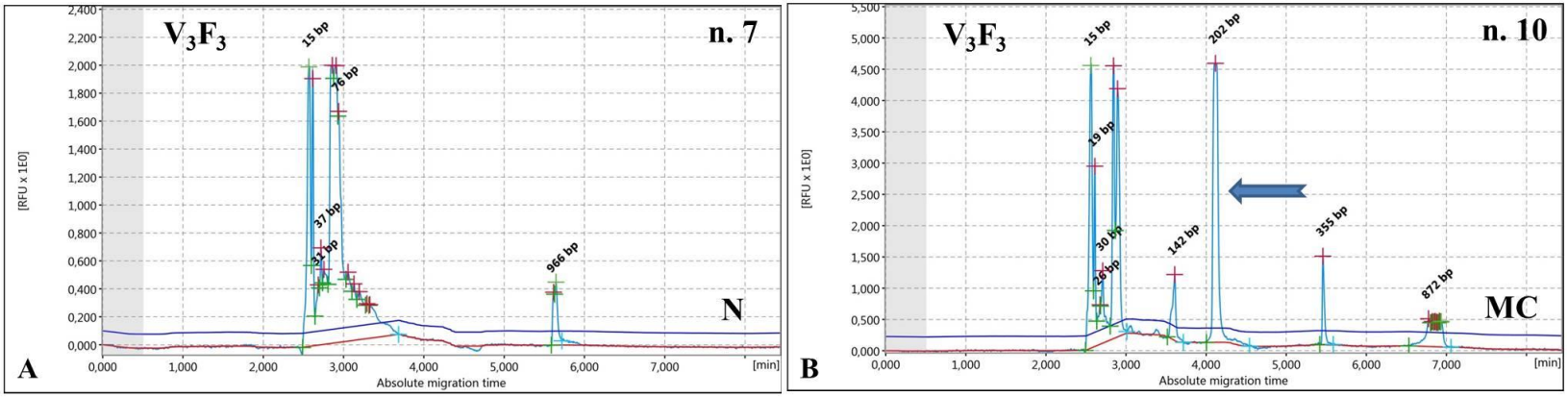


Fig 17C, Sample 33, V_3F_3 reaction interpreted as negative **(N); D, Sample 34**, V_3F_3 reaction interpreted as monoclonal **(MC)** for the presence of a peak of 292 bp.

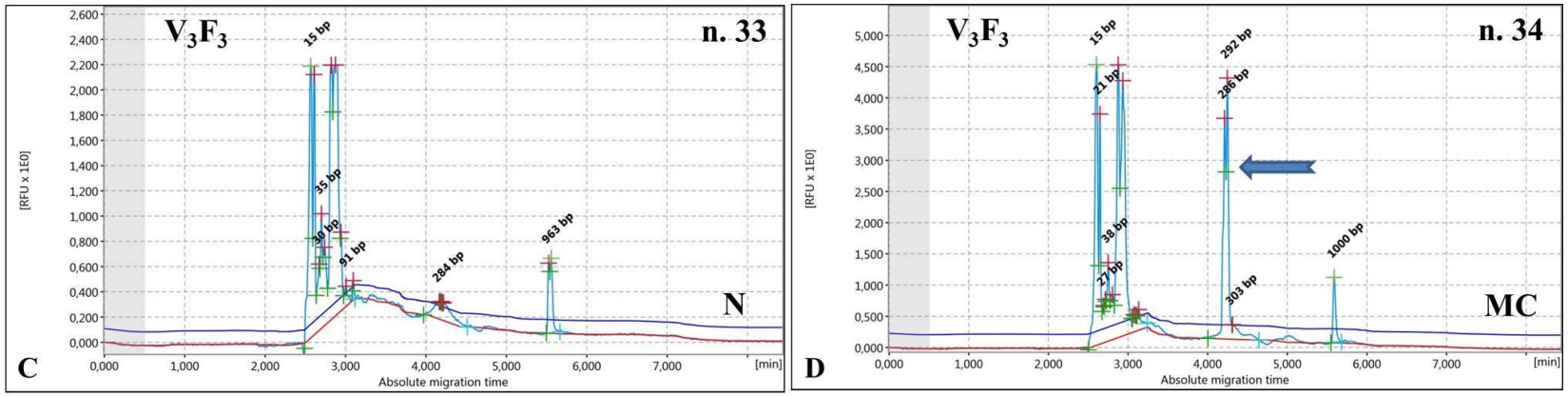


Figure 17. Continued.

Fig 17E, Sample 22, V_3F_3 reaction interpreted as polyclonal **(PC)** for the presence of multiple peaks (294bp) with Gaussian-curve like appearance

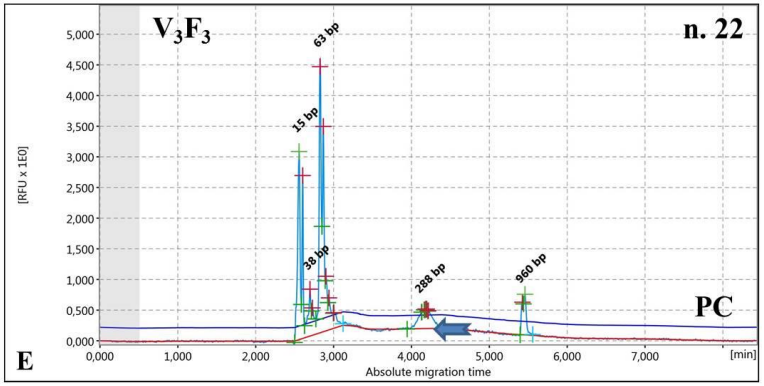
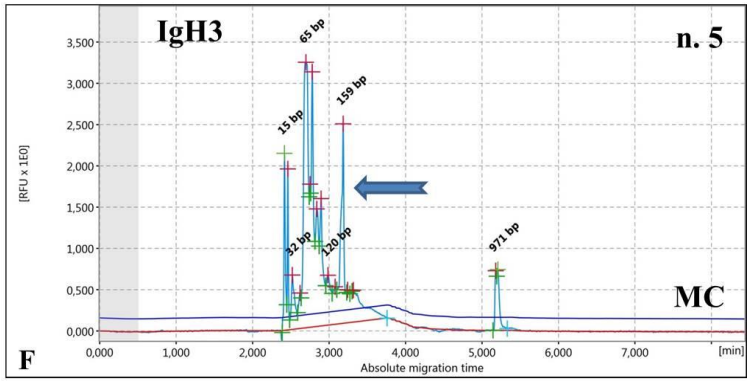


Fig 17F, Sample 5, IgH3 reaction interpreted as monoclonal **(MC)** for the presence of a peak ok 159 bp (arrow).NTC and positive cell line MS-4 were run along with the samples for both primers reactions(not. shown).



5.3.3.2.3. Capillary Electrophoresis (CE).

Amplification products using the IgH2 primer were run, if available, on capillary electrophoresis. The results from the 12 samples run (no. 3, 4, 5, 7, 10, 12, 15, 16, 17, 22, 25 both localisations, 32), were confirmed as either polyclonal or negative on the QIAxcel Advanced System.

Clonality using the primer V_3F_3 was investigated on capillary electrophoresis in all the samples previously tested on gels. The clonal result for case no. 10 (**Figure 17B**) was confirmed by the visualisation of a distinct peak of about 203bp, whereas case no. 34 (**Figure 17.D**) showed a distinct peak of 292bp in one sample, and two peaks of 292 and 286 bp in the duplicates, thus confirming the biclonal results given on page gel electrophoresis. Polyclonal results were evident in cases no. 12, 17, 22 (**Figure 17E**), 25 (both localisation), 29, 32. No specific product was identified for cases no. 5, 7 (**Figure 17A**), 15, 18, 24, 33 (**Figure 17C**), 36. Finally, pseudoclonal/polyclonal results were evident for the rest of the cases.

Some of the samples tested for IgH3 primer were also submitted to capillary electrophoresis. Results confirmed the ones obtained by PAGE: the only monoclonal results was sample no.5 with a distinct peak of 159bp (**Figure 17F**).

The multiplex (V_1F_2/V_3F_4) analysis was also run on the QIAxcel Advanced system, confirming the monoclonal appearance of sample no. 10 (peak of 187bp). All the rest of the samples were polyclonal (case no. 4, 5, 7, 10, 12, 15, 16, 17, 18, 22, 28, 29, 31, 32, 34) (**Figure 18.C**).

The singleplex reaction for V_3F_4 was run on the QIAxcel Advanced system, giving no specific products in all the run performed.

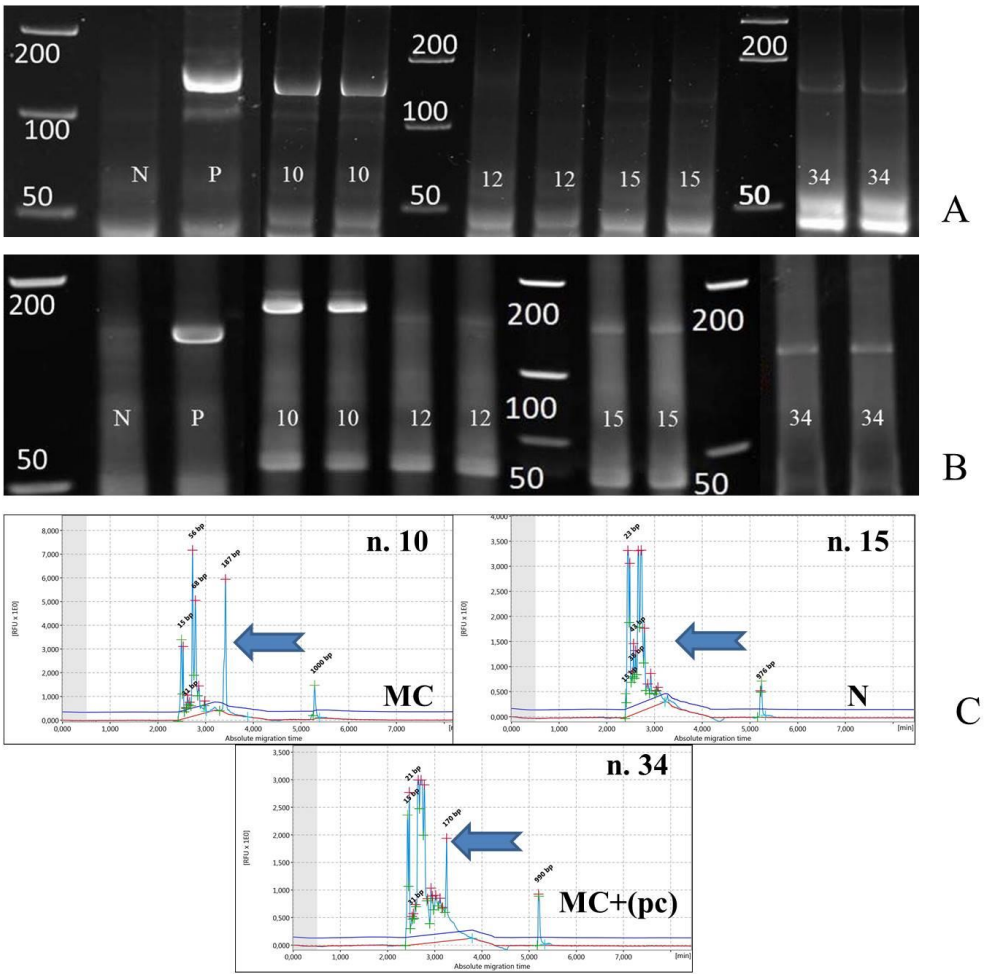


Figure 18. Agarose gel, PAGE and CE visualisation of PCR product, multiplex V_1F_2/V_3F_4 reaction.

Fig 18A, Agarose gel showing a multiplex reaction with V_1F_2 and V_3F_4 primer mix. **Sample 10**, interpreted as monoclonal for the presence of a single peak of 187 bp (arrow); **Sample 12,** interpreted as polyclonal; **Sample. 15,** interpreted as polyclonal; **Sample. 34,** interpreted as possible monoclonal; NTC and positive cell line MS-4 were run along with the samples.

Fig 18B, PAGE showing a multiplex reaction with V_1F_2 and V_3F_4 primer mix. **Sample 10**, interpreted as monoclonal; **Sample 12,** interpreted as polyclonal; **Sample. 15,** interpreted as polyclonal; **Sample. 34,** interpreted as possible monoclonal; NTC and positive cell line MS-4 were run along with the samples.

Fig 18C. Electropherograms of PCR product obtained from V_1F_2 and V_3F_4 primer mix amplification. **Sample 10,** confirmed as monoclonal **(MC)** for the presence of a single peak of 187bp (arrow); **sample no. 15,** confirmed as negative **(N)**; **sample no. 34**, interpreted as monoclonal with the polyclonal background **(MC+pc- arrow)** for the presence of a single peak of 170 bp (arrow) rising from multiple peaks with Gaussian-curve appearance. NTC and positive cell line MS-4 were run along with the samples (not shown).

5.3.4. COMPARISON OF THE ELECTROPHORESIS TECHNIQUES.

In general, the agreement between the three electrophoresis techniques was high. Considering the CE as the gold standard, sensitivity in detection of clonality in the single reactions ranged from 87% (TCRG reaction, both agarose and PAGE analysis) to 94% (V_3F_3 reaction for PAGE). Specificity was 100% for all the reactions. Few cases gave false positive results on gels. In particular, sample 5 amplified with TCR gave a bright band of difficult interpretation on agarose (**Figure 13**), which was apparent also on PAGE (**Figure 14**). However, CE confirmed the polyclonality of the sample (**Figure 15**). With the same reaction, an agarose run for sample 12 appeared to be clonal (**Figure 13**), but was confirmed as polyclonal by the other two analyses (PAGE, **Figure 14**; CE **Figure 15**); Moreover sample no.29 was defined as clonal on PAGE (**Figure 14**), but its polyclonality was confirmed on CE (**Figure 15**). Agarose gel interpretation for samples 16, 17 and 25 (both locations) was misleading: on Agarose, a bright band was visible, although polyclonality was confirmed on PAGE and CE.

Another disagreement between the three electrophoretic methods was observed in the V_3F_3 reaction for sample 22, which appeared clonal on Agarose gel (**Figure 16A**), but was confirmed as polyclonal on PAGE (**Figure 16B**) and CE (**Figure 17E**).

The clonality status of each sample (clonal vs polyclonal) was concordant in most of the cases between PAGE and CE, but the latter was able to give a better resolution of the results, making the distinction between pure monoclonal results and bi or oligoclonal results possible. PAGE was able to distinguish oligoclonal and biclonal result better than agarose in many cases (samples no. 7 and 15 for TCRG and sample 10 for V_3F_3 amplification). The Qiaxcel Advanced system allowed better characterisation of sample 34, amplified with V_3F_3 primer and the multiplex reaction for V_1F_2 and V_3F_4, also allowing the distinction of a clonal population within a polyclonal background in the latter reaction.

All the other results were in agreement between the three methods.

A summary of the results obtained by the three different methods is shown in **Table 16**.

Table 16. Comparison of the three electrophoresis methods. **AGAR,** TAE 4%LMP agarose, buffer, 200V 5 mins and 160V45 mins; **PAGE** Novex™ TBE Gels, 20%,180V, 90mins; **CE**, QIAxcel Advanced system with QIAxcel DNA High-Resolution Kit (Qiagen). **N-T**, T cell neoplasia; **N-B**, B cell neoplasia; **R**, reactive; **ND,** not a definitive diagnosis; **MC,** monoclonal; **BC,** biclonal; **OC,** oligoclonal; **PC,** polyclonal; **MC+pc,** monoclonal with polyclonal background; **N,** negative**; ?,** dubious interpretation, likely clonal; **blank,** electrophoresis not run.

		TCRG(80-120bp)			IgH2			V_3F_3 (210-280 bp)			IgH3			V_1F_2/V_3F_4 multiplex			V_3F_4 singleplex		
sample ID	FC immunophenotype	AGAR	PAGE	CE	AGAR	PAGE	CE	AGAR	PAGE	CE	AGAR	PAGE	CE	AGAR	PAGE	CE	AGAR	PAGE	CE
3	N-T	MC	MC	MC (140 bp)	N	PC	N	N	PC	N	N	N	not run	not run	not run	not run	not run	not run	not run
4	N-T	N	N	PC	PC	PC	PC	PC	PC	PC/PS	PC	N	not run	PC	N	PC	N	PC	N
5	N-B	MC	MC	PC	N	PC	N	N	PC	N	MC	MC	MC(158bp)	N	N	N	N	PC	N
7	T-N	MC	OC	OC (141/151/195 bp)	N	PC	N	PC	PC	N	not run	not run	not run	PC	PC	N	not run	not run	not run
10	N-B	PC	PC	PC/PS	PC	PC	N	C	BC	C (203 bp)	N	N	PC	MC	MC	MC(185bp)	not run	not run	not run
12	R	MC	PC	PC	PC	PC	pc	PC	PC	PC	not run	not run	not run	N	PC	not run	N	PC	N
15	N-T	MC	BC	BC (140/163 bp)	N	PC	N	N	PC	N	not run	not run	not run	PC	PC	PC	PC	PC	N
16	R	?	PC	PC	PC	PC	PC	PC	PC	PC/PS	PC	N	PC	N	N	?	N	PC	N
17	R	?	PC	PC	PC	PC	PC	PC	not run	PC	PC	N	PC	N	N	?	N	PC	N
18	B-N	N	N	not run	N	PC	not run	PC	not run	N	N	N	PC	PC	PC	PC	PC	PC	N
22	B-N	PC	PC	PC	N	PC	PC	C	N	PC	N	PC	PC	N	PC	PC	not run	not run	not run
24	B-N	PC	PC	not run	N	PC	not run	PC	PC	N	N	PC	PC/PS	N	PC	not run	not run	not run	not run
25s	R	?	PC	PC	PC	PC	PC	PC	N	PC	N	PC	PC	N	PC	?	N	PC	N
25l	R	?	PC	PC	PC	PC	not run	PC	N	PC	N	N	PC	N	?	?	N	PC	N
27	T-N	PC	PC	not run	N	PC	not run	PC	N	PC/PS	N	PC	PC	N	N	PC/PS	not run	not run	not run
28	ND	PC	PC	not run	N	PC	not run	PC	N	PC/PS	N	PC	PC	N	N	PC	not run	not run	not run
29	ND	PC	MC	PC	N	PC	not run	PC	N	PC	N	PC	not run	N	PC	PC	N	PC	N
31	T-N	PC	PC	PC	N	PC	not run	not run	not run	not run	N	PC	not run	N	N	PC	not run	not run	not run
32	R	PC	PC	PC	N	PC	PC	PC	PC	PC	N	PC	PC	N	N	PC/PS	not run	not run	not run
33	ND	PC	PC	?	PC	PC	not run	PC	PC	PC	N	PC	not run	PC	PC	not run	N	N	N
34	T-N	PC	PC	PC	N	PC	not run	C	C	BC (292/286bp)	N	PC	PC	?	?	MC+pc	N	PC	N
36	B-N	?	N	PC	N	PC	not run	PC	PC	N	N		not run	PC	N	PC	N	PC	N

5.3.5. ADDITIONAL ANALYSIS.

Reactive samples no. 16, 17, 18, 25 (both localisations), 29, 33, 36 were further analysed with other combinations of primers. Namely, the FR1 region of the IGHV1 gene was investigated using the V_1F_1 primer; The IGHV3 gene was examined in its FR1 region using two primers named V_3F_1 and V_3F_2. This reaction was set up in order to exclude the presence of any clonal rearranged B cell population and to confirm the diagnosis of reactive lymphocytic hyperplasia. All the reactions were run on Qiaxcel Advanced System and gave polyclonal results.

Moreover, given the availability of DNA for case no.34, we decided to investigate which of the five primers directed to the J region (J1, J2, J3, J4 and J5) of the V_3F_3 forward primer combination was responsible for the peak detected in the electropherogram. The combinations J1, J2, J4, J5 gave no clonal results (Figure 19A, B, D, E). A distinct peak of approximately 296bp was shown if the J3 reverse primer was used (Figure 19C). This sample was also submitted to a singleplex reaction for V_1F_2 and V_3F_4, due to the unclear appearance of the peaks of the multiplex analysis (Figure 19F). The Reaction carried out with primer V_3F_4 yielded a reproducible peak of approximately 284bp (Figure 19G). No peaks were visible for V_1F_2 amplification (Figure 19H).

In case no. 33 (immunophenotyped as a possible T cell neoplasia) the TCRG analysis gave a dubious result, as within the Gaussian curve made up from peaks of the same height, it was possible to identify a more prominent peak. The primer TCRG: this primer mix is designed for a multiplex reaction and contains four V primers (V1-2, V3, V4 and V5) and a mix of three J primers (J1, J2 and J3). Sample 33 was polyclonal for all the three rearrangements tested, excluding clonality for TCRG (Figure 20). Conversely, sample 15 showed clonality for both the primer combinations, showing a peak of approximately 135bp for J1 reverse primer combination, and a peak of approximately 128bp for the J2 combination, confirming the T- cell neoplasia. Both peaks retrieved seem to arise from a slightly more prominent peak (approximately 147bp) with a broad base: this could represent a small residual population of mixed lymphocytes, which gives the polyclonal background (Figure 21).

Figure 19. Additional reactions for IGH rearrangement analysis carried out for sample 34.

Fig19A, negative result for the singleplex reaction carried out with V_3F_3 forward primer and J1 reverse primer; **Fig19B,** negative result for the singleplex reaction carried out with V_3F_3 forward primer and J2 reverse primer; **Fig19C,** monoclonal (**MC**) result for the singleplex reaction carried out with V_3F_3 forward primer and J3 reverse primer: a peak of 296 bp is evident (arrow). **Fig19D,** a negative result for the singleplex reaction carried out with V_3F_3 forward primer and J4 reverse primer, **Fig19E,** a negative result for the singleplex reaction carried out with V_3F_3 forward primer and J5 reverse primer.

Figure 19A.

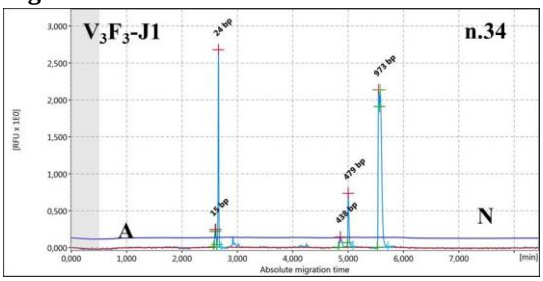


Figure 19B.

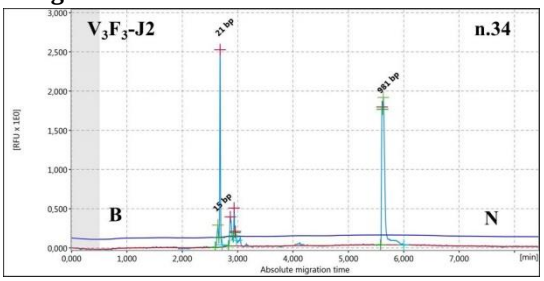


Figure 19C.

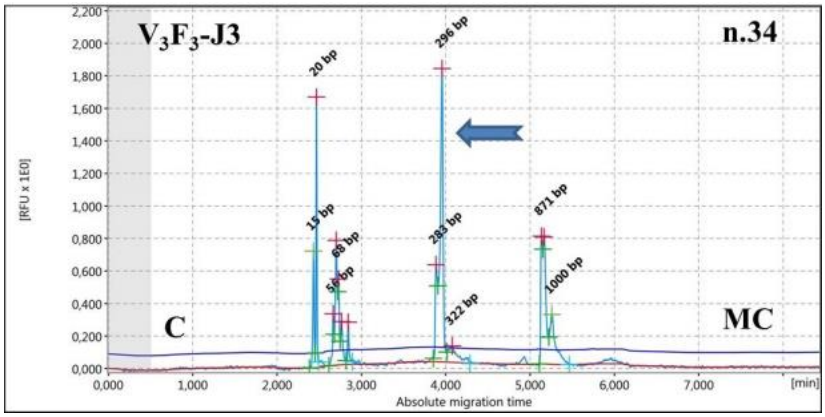


Figure 19D.

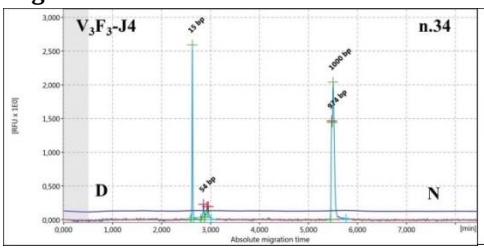


Figure 19E.

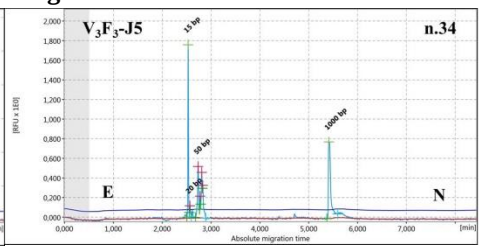


Figure 19. Continued.

Fig19F, the unclear result for the multiplex reaction carried out with V_1F_2-V_2F_4 primer combination. A peak of approximately 183bp can be individualised within multiple peaks of different sizes, rising the possibility of a clonal peak hidden by a polyclonal background.

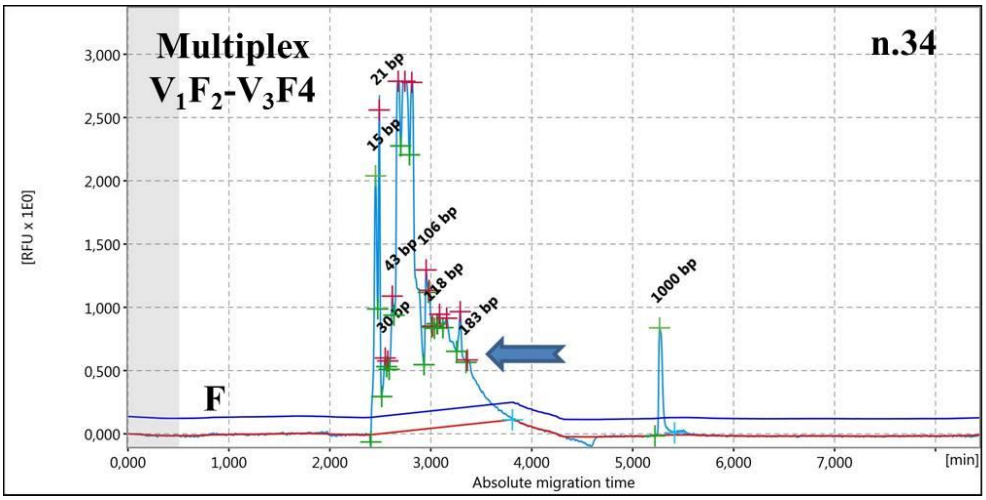


Fig19G, monoclonal **(MC)** result for the singleplex amplification using the sole primer V_3F_4.–

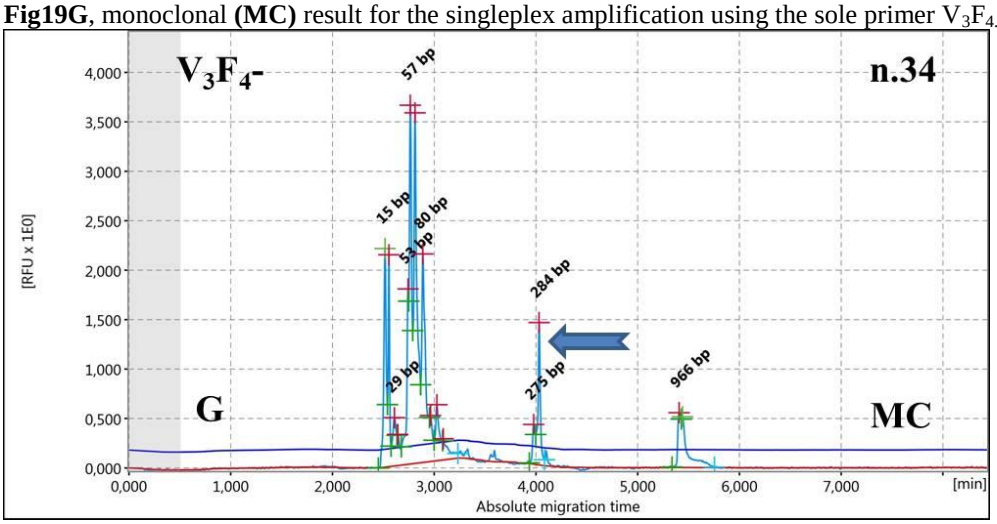


Fig19H, negative result of the singleplex amplification using the sole primer V_1F_2 for sample no.34

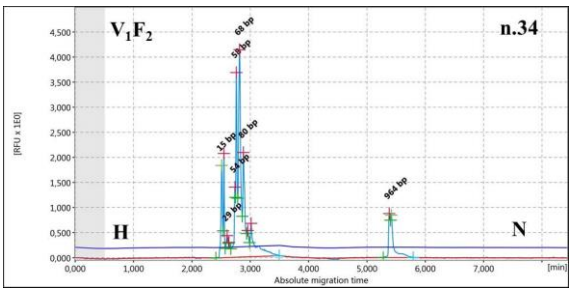


Figure 20. Additional reactions for TCR rearrangement analysis carried out for sample 33.

Fig 20A. Multiplex analysis of TCRG locus, using simultaneously the three reverse J primers. In the electropherograms, different peaks of the expected size are evident, raising the possibility of a clonal result;
Fig 20B, Fig 20C, Fig 20D negative results for the singleplex analysis of TCRG locus, ran using singularly J1, J2 and J3 reverse primer respectively. Clonality is thus excluded.

Figure 20A.

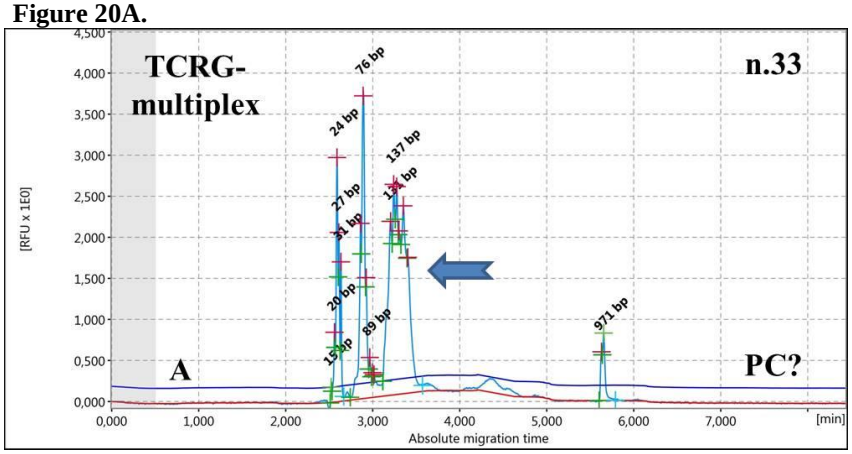


Figure 20B.

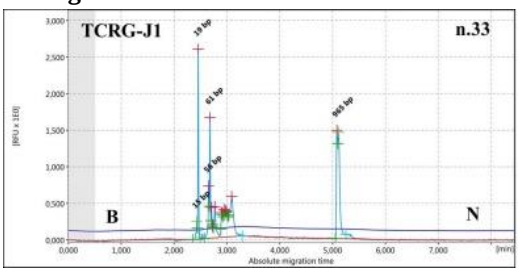


Figure 20C. .

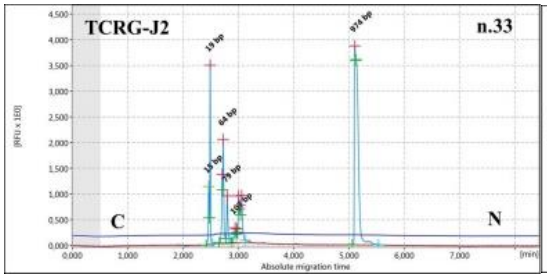


Figure 20D.

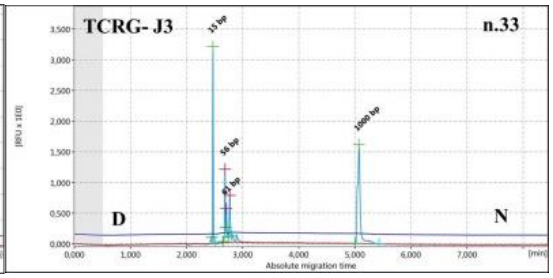


Figure 21. Additional reactions for TCR rearrangement analysis carried out for sample 15.
Fig21A, multiplex analysis of TCRG locus, using simultaneously the three reverse J primers. Detection of two peaks measuring 140bp and 163 bp (biclonal result, **BC**);

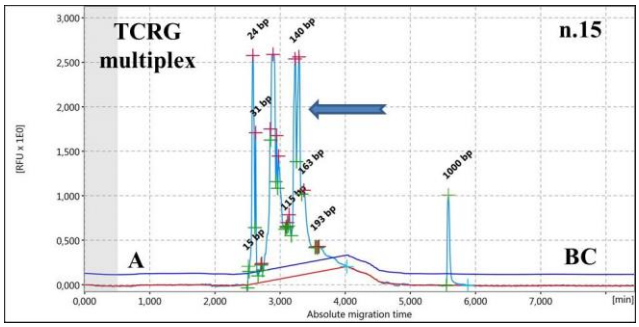


Fig21B, singleplex analysis of TCRG locus, ran using singularly J1reverse primer: detection of a single prominent peak of 133bp**;**

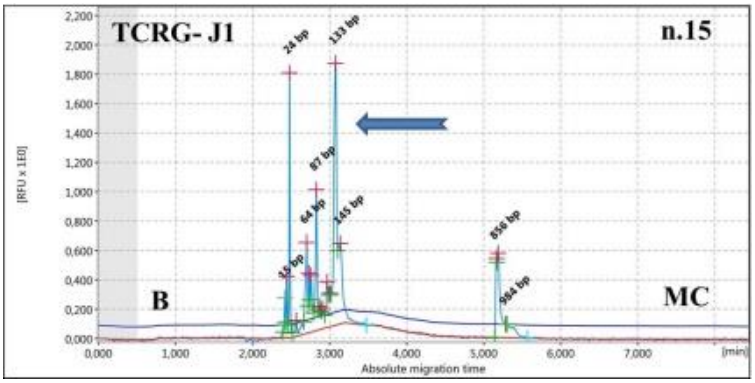
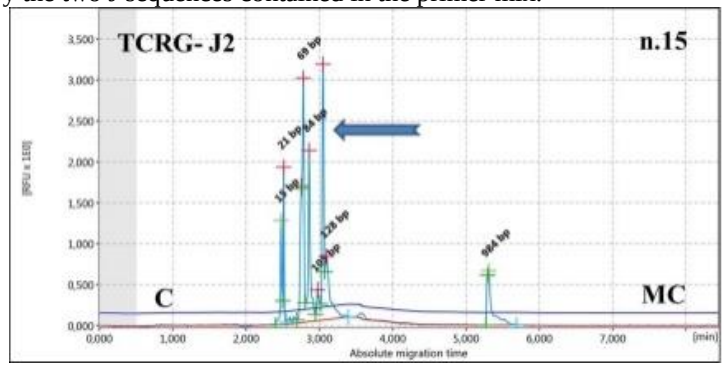


Fig21C, singleplex analysis of TCRG locus, ran using singularly J2 reverse primer: detection of a single prominent peak of biclonality could be due to the concurrent rearrangement of two TCRG genes, detected by the two J sequences contained in the primer mix.



5.3.6. FLOW CYTOMETRY VS PARR.

PARR failed to detect clonality in eight cases out of fourteen (57%) but was able to recognise all the reactive processes, with an overall agreement percentage of 62% (11 cases out of 21, if we consider both locations of the case 25 as a single case).

In four cases of T cell neoplasia (case no. 4, 27, 31, 33) clonal rearrangement of TCRG locus was not detectable. B lineage was not detected in four cases (case no. 18, 22, 24, 36). One case of T cell neoplasia rearranged clonally for IgH.

Three cases of T-cell neoplasia (case no. 3, 7, 15) were confirmed on PARR, whereas B cell neoplasia was confirmed in two cases (case no. 5 and 10). Interestingly, clonal results were retrieved in case 5, using the single IgH3 primer. In case 10, clonality was given by amplification of both V_3F_3 and the multiplex analysis of V_1F_2 and V_3F_4, thus impairing the ability to know which rearrangement was responsible for the clonal result.

Finally, six cases of reactive processes (case no.12, 16, 17, 25 both locations, 38, 32) on flow cytometry, were polyclonal on PARR: these cases were all tested for all the primers available, but one. In fact, case 28 was unfortunately not tested for the primer V_3F_3 as the insufficient sample was available: it would be interesting to have the whole panel run, as flow cytometry results were not definitive. In fact, despite the presence of a mixed population of T and B cells, an inversion in the proportion of CD4 and CD8 was evident within the T lymphocytes. This could be suggestive of an emerging CD8+ T cell lymphoma. Neoplasia, however, was not confirmed, but we cannot exclude the clonal rearrangement of the IGH gene (despite the T phenotype) which could possibly be picked up by the primer not tested.

The final comparison between the two techniques is illustrated in **Table 17**.

Table 17. PARR results and comparison to FC of the canine patients enrolled in the study. Flow cytometry and PARR results are indicated as Neoplastic (**N**) or Reactive (**R**); cell lineage is indicated as **T-N** (T cell neoplasia) or **B-N** (B cell neoplasia). If a question mark is present in the bracket (?), the most likely final diagnosis was the one indicated, but definitive diagnosis could not be achieved. Agreement between the two techniques is labelled in green; Disagreement is labelled in red.

SAMPLE ID	FLOW CYTOMETRY		AGREEMENT		PARR
3	T-N	N		N	T-N
5	B-N	N		N	B-N
7	T-N	N		N	T-N
15	T-N	N		N	T-N
10	B-N	N		N	B-N
34	T-N	N		N	B-N
4	T-N	N		R	NC
18	B-N	N		R	NC
22	B-N	N		R	NC
24	B-N	N		R	NC
27	T-N	N		R	NC
31	T-N	N		R	NC
33	T-N(?)	N		R	NC
36	B-N	N		R	NC
12	R			R	
16	R			R	
17	R			R	
25s	R			R	
25l	R			R	
28	R(?)			R	
29	R(?)			R	
32	R			R	

To summarise:

Of the twenty-two cases included in the study, PARR detected neoplasia in six of the fourteen neoplastic cases, while all the reactive processes were recognised as polyclonal by the assay.

PARR gave a diagnostic sensitivity of 63.5% and a specificity of 100%, as none of the reactive processes was diagnosed as neoplastic, giving a predictive values of a positive test of 100%. Conversely, this study shows that PARR did not agree with flow in eight cases, which were identified as neoplastic by flow showing that PARR has poor probability of diagnosing a true negative as negative.

The T phenotype was detected in three out of nine cases of T cell neoplasia. B phenotype was detected in two cases of B cell neoplasia out of six. In one case, clonal rearrangement of the TCRG locus was detected in a B cell neoplasia.

This result could be affected by the low concentration and poor quality of DNA amplified where flow cytometry was run on fresh cell samples

These results show how PARR should not be used as the sole method for lymphoma/leukaemia diagnosis but in conjunction with other modalities and the clinical condition of the cat.

5.4. DISCUSSION.

Our study was aimed to evaluate the ability of the clonality assay (PARR) to detect neoplasia and to compare it to immunophenotyping techniques such as flow cytometry in the course of feline lymphoproliferative diseases.

PARR was able to detect clonality in six cases out of fourteen (43%) with an overall sensitivity of 63.5% and a specificity of 100%. The primer set comprises one primer targeting TCRG gene rearrangements, made up from a combination of four forward and three reverse primers. This primer showed a sensitivity of 61.5% and a specificity of 100%, and in the original study[78] the use of these sequences, both in singleplex or multiplex reactions showed an overall sensitivity of 87%, with the more frequent detection of the rearrangement by the reverse primers J1 and J2. Therefore, B cell clonality was investigated in our study using primers retrieved from two different publications, for a total of five different primers targeting different regions of the IGH gene, and was able to detect neoplasia in 33% of the B cell neoplasia cases (overall sensitivity of 60% and specificity of 88.8%). The first article, published by Werner et al. in 2005[122], used the primers named IgH2 and IgH3, targeting the FR2 and FR3 of the IgHV3 gene respectively, were able to recognise B cell neoplasia in 68.2% of the cases. The second study[77] used multiplex reactions with a broader primer set directed to different segments of both IgHV1 and IgHV3, increasing the ability to detect neoplasia to 84%. Of these primer sets, which included a total of six primers, we selected the three most frequently rearranged (V_1F_2, V_3F_3, V_3F_4); we additionally ran the remaining three reactions to exclude B cell clonality if left-over DNA was available.

In this study, an extensive set of primers was tested, and we prefer to include primers from Mochizuki et al. (2011; 2012) for both for B and T cell clonality as they are considered a refinement of the previously published ones[27]. For this reason, we designed our primer set based mainly on Mochizuki publications, in conjunction with Werner's sequences. Other studies have tried to optimise and test these aforementioned primer sets. Sato et al. (2011)[187] tested Werner's primer, increasing the sensitivity to 89%. More recently, these primer sets have also been tested by Hammer et al. (2016)[27], which

demonstrated an overall sensitivity of 70%, increased to 90.1% for T cell neoplasia and 44% for B cell neoplasia (who still suffers from incomplete gene coverage). Our results are slightly different from the previously published ones with lower overall sensitivity and specificity.

Therefore, the significance of our results is hampered by the overall poor performance of the test, with the detection of neoplasia in a small percentage of neoplastic samples (less than half of the cases of confirmed neoplasia). In eight cases out of fourteen (57%), clonality was not detected, despite the diagnosis of neoplasia made by flow cytometry. PARR in this study did not detect either T or B cell lymphomas/ leukaemias.

As already mentioned in the previous chapter, failure in clonality detection can be due to technical or biological pitfalls. In the present study, the retrospective nature of the project and the use of cytological slides had a significant impact on the overall performance of PARR. Even though we tried to select samples stored for no longer than eighteen months, and although we tried to select the samples with higher cellularity and with more than one slide available, the overall success of the gDNA extraction was poor, and a vast number of samples were excluded due to the low DNA concentration and quality. Of the rest of the samples, only twelve (out of thirty-seven) had optimal DNA characteristics, according to the recently published recommendations[27], while nine were included nonetheless due to their good quality, despite the low gDNA concentrations. The presence of few B/T cells or DNA of poor quality[63] could explain why, in some cases, the signal was lacking, even though the amplification of the fAR as control gene was successful in all the samples. Thus, degradation of DNA, even if unlikely, cannot be entirely excluded.

On the other hand, cytological slides show fewer cells compared to fresh samples (tissue, blood or other kinds of fresh samples), and this could be an explanation for low DNA concentration and purity. Clearly, using slides instead of tissue samples shows advantages on many levels: firstly, collection of tissue samples requires a surgical approach, with higher cost and time for owners and surgeons; moreover, it is an invasive technique, requiring general anaesthesia and pain control therapy in most of the cases;

thirdly, the extraction of DNA from tissue samples can take up to two days, and requires the use of many toxic and irritating reagents (such as xylene). Conversely, cells for cytology can be obtained quickly and cost-efficiently, from conscious patients, in any practice, from Referral Centres to the small general practices. The samples can be submitted to the specialised laboratories and be archived while owners and oncologist consider additional diagnostic tests, and do not have special requirements for shipment and storage. However, especially for archived samples, the standardisation of the slide cellularity can be challenging: the quality and preservation of a cytological slide are strictly operator-dependent, and incorrect staining of the slides (mainly by inexperienced personnel) can impair the DNA quality and availability for molecular diagnostics. It has been reported that the minimum number of cells to perform clonality assays has to be approximately 50.000[37], and adequate yields can be achieved using cytological slides[188]. Shipping the sample in buffers if FNAs or the fresh blood sample in EDTA should be recommended, in order to assure adequate source material volumes. This way, cytological assessment and clonality could be performed efficiently by the laboratory and specific volumes of fresh samples containing a known number of cells can be submitted to PARR analysis.

Therefore, the low cellularity of a sample could potentially lead to the opposite result: if only a few amplifiable targets are present; even a polyclonal population can mimic a clonal one showing patterns that might look clonal. These results are defined as "pseudoclonal" and can be prevented by running the samples in duplicates or triplicates. Many studies recommend running each reaction in duplicate or triplicate[27,58], as a true clonal pattern will give the same result in both PCR reactions, and possible artefacts can be avoided. In our study, all the samples were run in duplicate, and none of them showed pseudoclonality.

A second explanation for the negative results obtained by PARR in our study is the possible use of non-functional primers. To ensure that our primers were working correctly , positive, negative and polyclonal controls were included as recomended[63,142] and run along with all the samples tested. We established and described our internal

positive controls (Chapter 3), selecting patients from our archive. However, we felt more confident to use well-established cell lines as positive controls: our patients' samples, in fact, were positive for some of the reaction tested, but our amplicons were not sequenced, so despite the size corresponding with the expected one, we could not feel confident about the exact rearrangement showed. We decided to include cell lines MS-4 and FT-1, kindly provided by Dr Hammer (University of Veterinary Medicine, Vienna, Austria). The first cell line, named MS-4 (RRID: CVCL_IW02)[124], was established from the pleural effusion of a nine years old FIV/FeLV negative Abyssinian cat with cutaneous B-cell lymphoma. In the original study, the cells were tested by immunocytochemistry and flow cytometry for correct immunophenotype and submitted to PARR analysis using previously described primer sets[74,122]. The cells showed clonal rearrangement for IGH gene but not for TCRG, confirming the B phenotype and were afterwards tested by the authors with the other designed primer set[77]. The second cell line, named FT-1 (RRID: CVCL_J508)[125] was obtained from a thymic lymphoma, tested for the TCRG targeting primers[78] and used as positive control in recent studies[27,189]. Using positive cell lines as controls enabled us to be confident about the correct functioning of our primers, excluding the event of incorrect annealing sequences to the target gene.

Another possibility for failure in clonality detection could be insufficient gene coverage and inadequate primer binding. Even though the primers used seemed to cover many segments of both TCRG and IGH gene, further unidentified V and J regions of both genes can be present and thus were not amplified by our primer set. In fact, a full description of the feline TCRG and IGH locus is still lacking, and the event of non-binding regions is a possibility.

Rearrangements of antigen receptor genes occur early in the differentiation of lymphocytes, making their detection a perfect target for PCR amplification and diagnosis of lymphoproliferative diseases, despite the maturation stage. Extensive studies about the feline genome and the frequency by which each gene is rearranged are still lacking,

impairing the development of a comprehensive and reliable primer set for the feline species.

Incomplete gene coverage can be attributable to the random nucleotide insertions and deletions that can occur throughout the antigen receptor genes. These mutations can develop not only in the CDRs but also in the FRs and in the primer binding sites[77,122] especially during the so-called "somatic hypermutation" (SHM) of IGHV segments that usually occur in antigen-stimulated B cells undergoing maturation in the germinal centers[143].

Moreover, especially in B cell neoplasia, chromosomal aberrations such as translocations or gene fusions can occur[190]: this is true in Human B cell neoplasms like follicular lymphomas and large B-cell lymphomas[191,192], where translocations involving *Ig* genes have been extensively described[193–195]. However chromosomal aberrations have also been described in the course of T cell neoplasia[196,197], except cutaneous neoplasms[198]. This chromosomal modification has been described as a possible etiological factor for malignant B-cell proliferations: one of the most extensively described is the c-myc gene fusion to the switch region of the IGH locus[199], and other translocations have been extensively described in a plethora of different malignant conditions[200]. Although some cytogenetic investigations have been carried out in the canine species[201–206], for the cat this is still a hard road. In fact, although c-myc gene activation and transduction has been recognised as an activator of the malignant proliferation of lymphocytes[207,208], especially after retroviral infections[209], chromosomal aberration ending in the modification of the AR genes has not been described.

To assure the maximum gene coverage by primers involved in PARR, in Human medicine, the BIOMED-2 Concerted Action tested vast combinations of primers, not only the heavy chain gene (including somatically mutated rearrangements) is targeted, but also the κ and the λ light chain encoding loci (IGK and IGL respectively)[95]. The TCR gene is targeted in its TCRB, TCRG, TCRD genes, although the latter could be omitted due to its complexity of interpretation[95,144]. An algorithm has been published in order to

decide how to proceed with performing clonality assays in human lymphoproliferative diseases[144].

In feline clonality assays, the primer set is still restricted to a small number of primers targeting only the IGH and the TCRG genes. However, recent advancements in the description of canine TCRA/TCRD locus and IGHL are promising[86], and studies on the feline genome are to be expected shortly. Moreover, the development of a TRD multiplex assay and an assay targeting the kappa- deleting element (KDE) for dogs, cats, and horses are in progress[58], promising an improvement in clonality assays for companion animals.

Another limitation of this study is the lack of clinical history and presentation of the patients as well as any follow-up. Especially in a scenario where immunophenotyping is still lacking an extensive panel of antibodies (compared to the dog), and where clonality assays are still not well established, a complete picture of the clinical case is mandatory. Due to the high possibility of false positive and false negative results and the limited data available on feline IGH and TCRG, any case submitted to advanced diagnostic techniques should be accompanied by the complete clinical history of the patient, to aid the most accurate diagnosis possible. Moreover, prospective studies, including not only diagnosis but also the follow-up could be useful in the definition of the importance of clonality assays in the feline species and possible prognostic value of the correlation between immunophenotype and rearrangement of the ARs loci.

Despite all the difficulties of performance and the pitfalls of the technique, PARR has a vast potential in terms of ability to identify a clonal population within a polyclonal one with high sensitivity. These results are encouraging showing how PARR could be useful in the early detection of Minimal Residual Disease (MRD) and lymphoma relapse, as is already done routinely in Human Oncology[210]. In the canine counterpart, studies on MRD by PARR have been attempted over the last ten years[211] with good results: the use of conventional PCR analysis[99,212] or real-time quantitative PCR (RT-qPCR)[213] have been shown to be not only an excellent diagnostic tool for lymphoma monitoring but also a useful prognostic indicator[98].

Studies on MRD detection for feline lymphomas have not been published so far, but quantitative sensitivity has been tested by different studies, in order to define the ability of published primers to detect clonality in the context of a polyclonal population. Mochizuki et al. (2012)[78] showed that when the neoplastic cells (namely derived from the T cell lymphoma FT-1)[125] were diluted into a healthy spleen, liver or duodenum, clonality was detected with 10% dilution as the lower limit. Similarly, Hammer et al. (2016)[27] performed serial dilutions of neoplastic B and T cells into polyclonal samples in order to test the qualitative and quantitative sensitivity of the primer set in use, which included Mochizuki's primers[77,78] and others[74]. The lower limit for detection of the clonal peak for T-cell lymphoma was 50%, while clonal rearrangements for the IGH gene was still visible within a dilution of 90% of the polyclonal lymphnode gDNA.

Further studies are required with this aim, and with careful selection of the cases (and possibly with a prospective design), followed by accurate detection of clonality and extensive sequencing. Next-generation sequencing (NGS), in fact, and design of patient's specific primers[214] could enhance the sensitivity of the detection of MRD for the Veterinary patient (feline or canine) and could at the same time deepen knowledge in IGH and TCRG loci rearrangements and help in the design of new primers (important especially in the feline species).

In the present study, three different electrophoretic methods were used to detect clonality. The overall poor performance of PARR does not allow full comparison of the techniques; however, in the few clonal cases, Agarose gel electrophoresis and PAGE were mostly concordant with CE. However, Agarose gel gave misdiagnosis in six samples for TCRG amplification, and in three samples for IGH amplification (one with primer V_3F_3 and two with the primer mix V_1F_2/V_3F_4). In these samples a bright band was visible, leading to the diagnosis of neoplasia, clarified subsequently as a reactive process on CE. Care should be taken in the interpretation of results on an agarose gel, as the poorer resolution could potentially show polyclonal populations as clonal.

Moreover, the separation of the bands in the cases of bi or oligoclonal samples was not efficient on Agarose but evident on PAGE. The latter technique, therefore, showed better

agreement to CE, with misdiagnosis only in four cases (two amplifications of TCRG and two amplifications of IGH with the same primer mix abovementioned). Most likely, the multiplex set of these primers, with a different combination of forward and reverse primers, could produce amplicons of similar size, which can overlap each other and show a polyclonal population as clonal (and vice versa).

For these reasons, CE has to be preferred to other visualisation techniques, as it can give superior resolution and the ability to distinguishing between peaks, even if differing by only a few base pairs in size. Moreover, as demonstrated by sample 34 in our case series, the distinction of the monoclonal population within a polyclonal background could be challenging by PAGE but could be facilitated by capillary electrophoresis, especially if primers are designed for a multiplex reaction. Additionally, the precise size determination could be used as a fingerprint of neoplastic cells in each case: monitoring the same neoplastic population during or after treatment or to determine if multiple lesions have the same neoplastic origin can be performed by capillary electrophoresis.

Ultimately, CE is an automated technique which has two significant advantages: it is cost and time effective, and eliminates the possible operator-dependent error in the interpretation of the results, especially for ambiguous cases. The use of CE is nowadays highly recommended[27], even mandatory, especially if PARR is run for diagnostic purposes for detection of primary cancers or MRD, and especially in ambiguous cases.

For instance, in our case series, a definitive diagnosis was not achievable by Flow Cytometry in two cases. In particular, case no. 29 showed a mixed population of B and T cells, and within this population, a small percentage of cells co-expressed CD4 and CD8, which is aberrant. When we performed PARR, and we read the results on PAGE, we diagnose the rearrangement of TCRG as clonal, due to the visualisation of two bands slightly smaller than 100bp, the expected size range. When this sample was subsequently run on CE, a polyclonal curve (measuring around 119bp), with a broad base and no distinct peak was visible, ruling out the diagnosis of T cell neoplasia. This represented an ambiguous case, where misdiagnosis can occur. Despite clinical information and clinical suspicion, and despite the indications by flow cytometry, PARR revealed on CE a

polyclonal population. In similar cases, it is recommended to re-run the PCR amplification in both the original specimen or on a freshly acquired one[144] in order to give a diagnosis that is as precise as possible. Unfortunately, the source material for this case was scant, and we could not repeat any DNA extraction or do any further PCR amplification. Moreover, we lack follow-up for this patient, so a final diagnosis is difficult to make. Finally, case 33 showed "A high proportion of cells that labelled with CD5 (T cell marker) with a low proportion of CD21 positive B cells (…). The majority of the T cells express CD4 and only a low proportion express CD8 (....).When gating the larger cells, the proportion of CD5+ CD4+ increased with an absence of B cells". This could be suggestive of T cell lymphoma, which was however excluded by PARR. In this case, in fact, multiplex PCR using the TCRG primer was performed: this reaction gave a dubious result, with the visualisation of a possible peak arising from the polyclonal background. However, when the singleplex analysis was run using the J reverse primers singularly, this event was excluded, and the sample was diagnosed as polyclonal. These cases point out how useful CE could be in the event of ambiguous cases, and how misdiagnosis can occur even using PAGE analysis.

A significant limitation of comparing immunophenotype by FC and clonality testing in the feline species is the limited knowledge of both techniques in this species. Research in feline lymphoma in fact, despite the high prevalence[8] of this neoplasm, is still limited, and this is true also for FC as a diagnostic tool. To our knowledge, the potential of FC in diagnosing and characterise feline lymphoproliferative diseases has been elucidated in three studies[25,33,215], with different panels of monoclonal antibodies.

Flow Cytometry analysis presents many limitations in cat diagnostics. First, the mAbs panel in the feline species is still restricted when compared to the dog[216], although several attempts have been made to find more mAbs by the cross-species validation of antibodies[217–219]. Some of the key markers in canine lymphoma diagnostics in fact are not available in the cat: for example, CD34, expressed by blasts, is not available in the feline species, impairing the possibility of recognition of more aggressive diseases, as in dogs.

Additionally, not all the antibodies validated can be useful for immunodiagnostics, and it would be useful to consider even in the feline species multiple simultaneous labelling, which so far has not been investigated[33], as the same marker can potentially mark cells of different lineages.

It has been reported as well that the high prevalence of non FeLV-related intra-abdominal lesions seems to be more frequent nowadays[220] making the acquisition of cellular specimens by FNA difficult. Cellularity of the sample in fact is important for the high performance of the technique: in the event of low cellularity samples, the mAbs panel has to be restricted[215] and reducing a panel which is already limited, increases the risk of incorrect diagnosis. However, Martini et al.[215] showed that the cellular concentration and performance of the technique are not affected by size and site of the lesion, making FC a suitable technique in cats, even if affected by challenging to reach and sample lesions. The sampling technique and the conservation of the specimens also seem to affect the quality of the diagnosis: large needles (21gauge) are more likely to give good quality samples, but the FC analysis has to be conducted within 48h from collection[33], making the application of this technique not always feasible.

Significant limitations of the study, impairing the objective judgement of the comparison between the two techniques. Due to the retrospective nature of the study, we had access only to the FC reports: we did not have access to the original plots and protocols, and the sampling technique, the processing and the gating procedure are unknown. Therefore, we cannot exclude the possibility of FC misdiagnosis. We expect, in the near future, to establish prospective studies, with careful selection of the patients, accurate data collection and accurate sample processing, both for FC analysis and clonality assays.

These cases show how important it is to have available a comprehensive set of antibodies for FC and primers for PARR. If neoplasia is suspected all the primer combinations possible have to be run if the expected result is not achieved, and in-depth knowledge of molecular biology is mandatory. Inexperienced personnel should not run clonality testing, and a molecular biologist has to accompany the clinical pathologist on the path to the final diagnosis. Clonality testing is a useful, powerful tool in lymphoma

diagnostics, but results have to be interpreted with an in-depth knowledge of all the facts, in order to provide the best verdict possible.

5.5. CONCLUSIONS.

In conclusion, despite the overall poor performance of the clonality testing of this study, we can conclude the following.

PARR is a powerful technique in the assessment of clonality in the course of lymphoproliferative diseases. Nevertheless, some precautions have to be taken when this assay is performed. Cytological slides are suitable source material for gDNA, easily obtainable in any practice and easily transportable, even though caution in choosing specimens has to be taken: highly cellular samples and more than one slides have to be preferred for extraction, to assure sufficient DNA yield of functional purity. Preliminary quality control of the extracted DNA is mandatory: spectrophotometry can meet this aim, but the amplification of a control gene is the best method to check eventual DNA degradation.

Additionally, pseudoclonality due to insufficient target DNA has to be prevented by the performance of PCR in duplicate or triplicate.

Primer sets have to be the most comprehensive possible, to avoid possible insufficient gene coverage: when neoplasia is strongly suspected, the use of different primers from the original routine primer sets has to be included. In the case of primers designed for multiplex assays, the possibility of running singleplex assays using single combinations of forward and reverse primers have to be considered. This would potentially allow distinguishing between true clonal and polyclonal cell populations.

Samples have to be run along with positive and negative controls. Cell lines or well established positive controls have to be preferred, in order to check the functionality of primers and the reaction in total.

Automated detection techniques have to be preferred to the old gel electrophoresis. Despite the good concordance between PAGE and CE visualisation methods, the cost-

time effectiveness and the accuracy of CE detected clonality with high resolution, makes this technique essential when clonality testing has to be carried out. Automated capillary electrophoresis can confidently distinguish between polyclonal and true clonal populations in most of the cases, especially in ambiguous cases.

Further studies are thus required: due to the incomplete knowledge about the feline genome, extensive sequencing and primer design are desirable in the near future. This could lead to a better understanding of AR genes rearrangements and cancer aetiology, with better characterisation of lymphoproliferative diseases in companion animals. Describing such genetic changes could lead to a better understanding of diagnosis, prognosis and management of lymphoproliferative disease in companion animals and in the cat, which is frequently affected by such disorders.

Finally, interpretation of the results by the clinical pathologist has to be framed within a highly interactive group, comprising clinicians, molecular biologists, pathologists, haematologists, and immunologists. This model has to exist and be active in all the facilities offering PARR as a diagnostic tool, allowing the best possible diagnosis and management of the veterinary oncology patient.

References

1. Valli, V. E. Veterinary Comparative Hematopathology. *Vet. Comp. Hematop.* 1–558 (2008). doi:10.1002/9780470344545

2. Withrow, S. J. & Vail, D. M. *Small Animal Clinical Oncology*. (2007).

3. Marconato, L., Gelain, M. E. & Comazzi, S. The dog as a possible animal model for human non-Hodgkin lymphoma: a review. *Hematol. Oncol.* **31,** 1–9 (2013).

4. Gabor, L. J., Malik, R. & Canfield, P. J. Clinical and anatomical features of lymphosarcoma in 118 cats. *Aust. Vet. J.* **76,** 725–732 (1998).

5. Gabor, L. J., Canfield, P. J. & Malik, R. Immunophenotypic and histological characterisation of 109 cases of feline lymphosarcoma. *Aust. Vet. J.* **77,** 436–441 (1999).

6. Vail, D. M., Moore, A. S., Ogilvie, G. K. & Volk, L. M. Feline lymphoma (145 cases): proliferation indices, cluster of differentiation 3 immunoreactivity, and their association with prognosis in 90 cats. *J. Vet. Intern. Med.* **12,** 349–354 (1998).

7. Vezzali, E., Parodi, A. L., Marcato, P. S. & Bettini, G. Histopathologic classification of 171 cases of canine and feline non-Hodgkin lymphoma according to the WHO. *Vet. Comp. Oncol.* **8,** 38–49 (2010).

8. Louwerens, M., London, C. a, Pedersen, N. C. & Lyons, L. a. Feline lymphoma in the post-feline leukemia virus era. *J. Vet. Intern. Med.* **19,** 329–335 (2005).

9. Owen, L. TNM Classification of tumours in domestic animals. *World Heal. Organ.* 1–52 (1980).

10. Vail, D. M., Moore, a S., Ogilvie, G. K. & Volk, L. M. Feline lymphoma (145 cases): proliferation indices, cluster of differentiation 3 immunoreactivity, and their association with prognosis in 90 cats. *J. Vet. Intern. Med.* **12,** 349–354 (2015).

11. Mooney, S. C. *et al.* Treatment and prognostic factors in lymphoma in cats: 103 cases (1977-1981). *J. Am. Vet. Med. Assoc.* **194,** 696–702 (1989).

12. Schiffman, J. D. & Breen, M. Comparative oncology: what dogs and other species can teach us about humans with cancer. *Philos. Trans. R. Soc. B Biol. Sci.* **370,** 20140231 (2015).

13. Cogliatti, S. B. & Schmid, U. Who is WHO and what was REAL? *Swiss Med. Wkly.* **132,** 607–17 (2002).

14. Parodi, a L. Classification of Malignant Lymphoma in Domestic Animals: History and Conceptual Evolution. *Eur. J. Vet. Pathol.* **7,** 43–50 (2001).

15. CHINO, J. *et al.* Cytomorphological and Immunological Classification of Feline Lymphomas: Clinicopathological Features of 76 Cases. *J. Vet. Med. Sci.* **75,** 701–707 (2013).

16. Ponce, F. *et al.* A morphological study of 608 cases of canine malignant lymphoma in France with a focus on comparative similarities between canine and human lymphoma morphology. *Vet. Pathol.* **47,** 414–433 (2010).

17. Valli, V. E. *et al.* Classification of Canine Malignant Lymphomas According to the World

Health Organization Criteria. *Vet. Pathol.* **48,** 198–211 (2011).

18. Appelbaum, F. R. *et al.* Phenotyping of canine lymphoma with monoclonal antibodies directed at cell surface antigens: classification, morphology, clinical presentation and response to chemotherapy. *Hematol. Oncol.* **2,** 151–168 (1984).

19. Carter, R. F., Valli, V. E. & Lumsden, J. H. The cytology, histology and prevalence of cell types in canine lymphoma classified according to the National Cancer Institute Working Formulation. *Can. J. Vet. Res.* **50,** 154–164 (1986).

20. Greenlee, P. G. *et al.* Lymphomas in dogs. A morphologic, immunologic, and clinical study. *Cancer* **66,** 480–490 (1990).

21. Valli, V. E. *et al.* The histologic classification of 602 cases of feline lymphoproliferative disease using the National Cancer Institute working formulation. *J. Vet. Diagnostic Investig.* **12,** 295–306 (2000).

22. Bennett, J. M. *et al.* Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br. J. Haematol.* **33,** 451–458 (1976).

23. Bene, M. C. *et al.* Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia* **9,** 1783–1786 (1995).

24. Jain, N. C. *et al.* Proposed criteria for classification of acute myeloid leukemia in dogs and cats. *Vet. Clin. Pathol.* **20,** 63–82 (1991).

25. Dean, G. A., Groshek, P. M., Jain, N. C. & Hoover, E. A. Immunophenotypic analysis of feline haemolymphatic neoplasia using flow cytometry. *Comp. Haematol. Int.* **5,** 84–92 (1995).

26. Cheson, B. D. *et al.* Recommendations for initial evaluation, staging, and response assessment of hodgkin and non-hodgkin lymphoma: The lugano classification. *J. Clin. Oncol.* **32,** 3059–3067 (2014).

27. Hammer, S. E. *et al.* Characterization of a PCR-based lymphocyte clonality assay as a complementary tool for the diagnosis of feline lymphoma. *Vet. Comp. Oncol.* 1–16 (2016). doi:10.1111/vco.12277

28. Eichenauer, D. A. *et al.* Hodgkin lymphoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. **0,** 1–11 (2018).

29. Stewart, C. J., Duncan, J. A., Farquharson, M. & Richmond, J. Fine needle aspiration cytology diagnosis of malignant lymphoma and reactive lymphoid hyperplasia. *J Clin Pathol* **51,** 197–203 (1998).

30. Young, N. A., Al-Saleem, T. I., Ehya, H. & Smith, M. R. Utilization of fine-needle aspiration cytology and flow cytometry in the diagnosis and subclassification of primary and recurrent lymphoma. *Cancer* **84,** 252–261 (1998).

31. Cozzolino, I., Rocco, M., Villani, G. & Picardi, M. Lymph node fine-needle cytology of non-Hodgkin lymphoma: Diagnosis and classification by flow cytometry. *Acta Cytol.* **60,** 302–314 (2016).

32. Comazzi, S. & Gelain, M. E. Use of flow cytometric immunophenotyping to refine the cytological diagnosis of canine lymphoma. *Vet. J.* **188,** 149–155 (2011).

33. Guzera, M., Cian, F., Leo, C., Winnicka, A. & Archer, J. The use of flow cytometry for immunophenotyping lymphoproliferative disorders in cats: a retrospective study of 19 cases. *Vet. Comp. Oncol.* **14,** 40–51 (2016).

34. Wolfesberger, B. *et al.* Does categorisation of lymphoma subtypes according to the World Health Organization classification predict clinical outcome in cats? *J. Feline Med. Surg.* **19,** 897–906 (2017).

35. Wilkerson, M. J. *et al.* Lineage differentiation of canine lymphoma/leukemias and aberrant expression of CD molecules. *Vet. Immunol. Immunopathol.* **106,** 179–196 (2005).

36. Sueiro, F. A. R., Alessi, A. C. & Vassallo, J. Canine lymphomas: a morphological and immunohistochemical study of 55 cases, with observations on p53 immunoexpression. *J. Comp. Pathol.* **131,** 207–213 (2004).

37. Burkhard, M. J. & Bienzle, D. Making Sense of Lymphoma Diagnostics in Small Animal Patients. *Clin. Lab. Med.* **35,** 591–607 (2015).

38. Stokol, T., Nickerson, G. A., Shuman, M. & Belcher, N. Dogs with Acute Myeloid Leukemia Have Clonal Rearrangements in T and B Cell Receptors. *Front. Vet. Sci.* **4,** (2017).

39. Vernau, W. & Moore, P. F. An immunophenotypic study of canine leukemias and preliminary assessment of clonality by polymerase chain reaction. *Vet. Immunol. Immunopathol.* **69,** 145–164 (1999).

40. Workman, H. C. & Vernau, W. Chronic lymphocytic leukemia in dogs and cats: The veterinary perspective. *Vet. Clin. North Am. - Small Anim. Pract.* **33,** 1379–1399 (2003).

41. Williams, M. J. *et al.* Canine Lymphopr oli fer ative Dis eas e Characteriz ed by Lymphocytosis: Immunophe n o t y p i c Ma r k e r s of P r o g n o s i s. 596–601 (2008).

42. Cian, F. *et al.* Stability of immunophenotypic lymphoid markers in fixed canine peripheral blood for flow cytometric analysis. *Vet. Clin. Pathol.* **43,** 101–108 (2013).

43. Ponce, F. *et al.* Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy. *Vet. J.* **167,** 158–166 (2004).

44. Fabre-Guillevin, E. *et al.* Aggressive non-Hodgkin's lymphoma: Concomitant evaluation of interleukin-2, soluble interleukin-2 receptor, interleukin-4, interleukin-6, interleukin-10 and correlation with outcome. *Leuk. Lymphoma* **47,** 603–611 (2006).

45. Frantz, A. M. *et al.* Molecular profiling reveals prognostically significant subtypes of canine lymphoma. *Vet. Pathol.* **50,** 693–703 (2013).

46. Valli, V. E., Kass, P. H., Myint, M. S. & Scott, F. Canine Lymphomas: Association of Classification Type, Disease Stage, Tumor Subtype, Mitotic Rate, and Treatment With Survival. *Vet. Pathol.* **50,** 738–748 (2013).

47. Novacco, M. *et al.* Prognostic factors in canine acute leukaemias: a retrospective study. *Vet. Comp. Oncol.* **14,** 409–416 (2016).

48. Zandvliet, M. Canine lymphoma: a review. *Vet. Q.* **36,** 76–104 (2016).

49. Teske, E., Van Straten, G., Van Noort, R. & Rutteman, G. R. Chemotherapy with cyclophosphamide, vincristine, and prednisolone (COP) in cats with malignant lymphoma: New results with an old protocol. *J. Vet. Intern. Med.* **16,** 179–186 (2002).

50. Simon, D., Eberle, N., Laacke-Singer, L. & Nolte, I. Combination chemotherapy in feline lymphoma: treatment outcome, tolerability, and duration in 23 cats. *J. Vet. Intern. Med.* **22,** 394–400 (2008).

51. Collette, S. A. *et al.* Treatment of feline intermediate- to high-grade lymphoma with a modified university of Wisconsin-Madison protocol: 119 cases (2004-2012). *Vet. Comp. Oncol.* **14,** 136–146 (2016).

52. SATO, H. *et al.* Prognostic Analyses on Anatomical and Morphological Classification of Feline Lymphoma. *J. Vet. Med. Sci.* **76,** 807–811 (2014).

53. Moore, P. F., Rodriguez-Bertos, A. & Kass, P. H. Feline Gastrointestinal Lymphoma: Mucosal Architecture, Immunophenotype, and Molecular Clonality. *Vet. Pathol.* **49,** 658–668 (2012).

54. Patterson-Kane, J. C., Kugler, B. P. & Francis, K. The possible prognostic significance of immunophenotype in feline alimentary lymphoma: A pilot study. *J. Comp. Pathol.* **130,** 220–222 (2004).

55. Chabanne, L. *et al.* A canine granular lymphocyte proliferative disease without an aggressive clinical course. *J. Vet. Intern. Med.* **15,** 249–251 (2001).

56. Kiupel, M. *et al.* Diagnostic Algorithm to Differentiate Lymphoma From Inflammation in Feline Small Intestinal Biopsy Samples. *Vet. Pathol.* **48,** 212–222 (2011).

57. van Dongen, J. J. M. *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* **17,** 2257–2317 (2003).

58. Keller, S. M., Vernau, W. & Moore, P. F. Clonality Testing in Veterinary Medicine: A Review With Diagnostic Guidelines. *Vet. Pathol.* **53,** 711–725 (2016).

59. Fivenson, D. P., Saed, G. M., Beck, E. R., Dunstan, R. W. & Moore, P. F. T-cell receptor gene rearrangement in canine mycosis fungoides: further support for a canine model of cutaneous T-cell lymphoma. *J. Invest. Dermatol.* **102,** 227–230 (1994).

60. Momoi, Y. *et al.* Establishment and characterization of a canine T-lymphoblastoid cell line derived from malignant lymphoma. *Vet. Immunol. Immunopathol.* **59,** 11–20 (1997).

61. Spagnolo, D. V. *et al.* The role of molecular studies in lymphoma diagnosis: A review. *Pathology* **36,** 19–44 (2004).

62. Wainscoat, J. S. & Fey, M. F. Assessment of Clonality in Human Tumors : A Review Assessment of Clonality in Human Tumors : A Review. 1355–1360 (1990).

63. Langerak, A. W. *et al.* EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. *Leukemia* **26,** 2159–2171

(2012).

64. Burnett, R. C. *et al.* Diagnosis of canine lymphoid neoplasia using clonal rearrangements of antigen receptor genes. *Vet. Pathol.* **40,** 32–41 (2003).

65. Massari, S. *et al.* The deduced structure of the T cell receptor gamma locus in Canis lupus familiaris. *Mol. Immunol.* **46,** 2728–2736 (2009).

66. Bao, Y., Guo, Y., Xiao, S. & Zhao, Z. Molecular characterization of the VH repertoire in Canis familiaris. *Vet. Immunol. Immunopathol.* **137,** 64–75 (2010).

67. Tamura, K. *et al.* Development of the polymerase chain reaction assay based on the canine genome database for detection of monoclonality in B cell lymphoma. *Vet. Immunol. Immunopathol.* **110,** 163–167 (2006).

68. Yagihara, H. *et al.* Genomic organization of the T-cell receptor γ gene and PCR detection of its clonal rearrangement in canine T-cell lymphoma/leukemia. *Vet. Immunol. Immunopathol.* **115,** 375–382 (2007).

69. Jeon, S., Lee, M. J., Park, J. & Kang, S. H. Fast molecular diagnostics of canine T-cell lymphoma by PCR and capillary gel electrophoresis with laser-induced fluorescence detector. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **854,** 268–272 (2007).

70. Gentilini, F., Calzolari, C., Turba, M. E., Bettini, G. & Famigli-Bergamini, P. GeneScanning analysis of Ig/TCR gene rearrangements to detect clonality in canine lymphomas. *Vet. Immunol. Immunopathol.* **127,** 47–56 (2009).

71. Chaubert, P. *et al.* Improved polymerase chain reaction – based method to detect early- stage epitheliotropic T-cell lymphoma (mycosis fungoides) in formalin-fixed , paraffin-embedded skin biopsy specimens of the dog. **29,** 20–29 (2010).

72. Keller, S. M. & Moore, P. F. A novel clonality assay for the assessment of canine T cell proliferations. *Vet. Immunol. Immunopathol.* **145,** 410–419 (2012).

73. Werner, J. A. *et al.* Characterization of feline immunoglobulin heavy chain variable region genes for the molecular diagnosis of B-cell neoplasia. *Vet. Pathol.* **42,** 596–607 (2005).

74. Henrich, M., Hecht, W., Weiss, A. T. & Reinacher, M. A new subgroup of immunoglobulin heavy chain variable region genes for the assessment of clonality in feline B-cell lymphomas. *Vet. Immunol. Immunopathol.* **130,** 59–69 (2009).

75. Weiss, A. T. A., Hecht, W., Henrich, M. & Reinacher, M. Characterization of C-, J- and V-region-genes of the feline T-cell receptor ?? *Vet. Immunol. Immunopathol.* **124,** 63–74 (2008).

76. Weiss, A. T. H. A., Klopfleisch, R. & Gruber, A. D. T-Cell Receptor γ Chain Variable and Joining Region Genes of Subgroup 1 are Clonally Rearranged in Feline B- and T-Cell Lymphoma. *J. Comp. Pathol.* **144,** 123–134 (2011).

77. Mochizuki, H. *et al.* Multiplex PCR and Genescan analysis to detect immunoglobulin heavy chain gene rearrangement in feline B-cell neoplasms. *Vet. Immunol. Immunopathol.* **143,** 38–45 (2011).

78. Mochizuki, H. *et al.* GeneScan analysis to detect clonality of T-cell receptor γ gene rearrangement in feline lymphoid neoplasms. *Vet. Immunol. Immunopathol.* **145,** 402–409

(2012).

79. Valli, V. E., Vernau, W., De Lorimier, L. P., Graham, P. S. & Moore, P. F. Canine indolent nodular lymphoma. *Vet. Pathol.* **43,** 241–256 (2006).

80. Waugh, E. M. *et al.* Optimisation and validation of a PCR for antigen receptor rearrangement (PARR) assay to detect clonality in canine lymphoid malignancies. *Vet. Immunol. Immunopathol.* **182,** 115–124 (2016).

81. Chaubert, P. *et al.* Improved Polymerase Chain Reaction–Based Method to Detect Early-Stage Epitheliotropic T-Cell Lymphoma (Mycosis Fungoides) in Formalin-Fixed, Paraffin-Embedded Skin Biopsy Specimens of the Dog. *J. Vet. Diagnostic Investig.* **22,** 20–29 (2010).

82. McGavin, J. F. Z. M. D. *Pathologic Basis of Veterinary Disease*. (2017).

83. Sciammas, R., Tatsumi, Y., Sperling, A. I., Arunan, K. & Bluestone, J. A. TCR gamma delta cells: mysterious cells of the immune system. *Immunol.Res.* **13,** 268–279 (1994).

84. Blom, B. B. *et al.* TCR Gene Rearrangements and Expression of the Pre-T Cell Receptor Complex During Human T-Cell Differentiation. 3033–3043 (1999).

85. Keller, S. M. & Moore, P. F. Rearrangement patterns of the canine TCRγ locus in a distinct group of T cell lymphomas. *Vet. Immunol. Immunopathol.* **145,** 350–361 (2012).

86. Martin, J. *et al.* Comprehensive annotation and evolutionary insights into the canine (Canis lupus familiaris) antigen receptor loci. *Immunogenetics* **70,** 223–236 (2018).

87. Moore, P. F., Woo, J. C., Vernau, W., Kosten, S. & Graham, P. S. Characterization of feline T cell receptor gamma (TCRG) variable region genes for the molecular diagnosis of feline intestinal T cell lymphoma. *Vet. Immunol. Immunopathol.* **106,** 167–178 (2005).

88. Weiss, A. T. A., Hecht, W. & Reinacher, M. Feline T-Cell Receptor gamma V- and J-Region Sequences Retrieved from the Trace Archive and from Transcriptome Analysis of Cats. *Vet. Med. Int.* **2010,** 953272 (2010).

89. Schroeder, H. W. J. & Cavacini, L. Structure and Function of Immunoglobulins (author manuscript). *J. Allergy Clin. Immunol.* **125,** S41–S52 (2010).

90. Werner, J. A. *et al.* Characterization of Feline Immunoglobulin Heavy Chain Variable Region Genes for the Molecular Diagnosis of B-cell Neoplasia. *Vet. Pathol.* **42,** 596–607 (2005).

91. Henrich, M., Hecht, W., Weiss, A. T. & Reinacher, M. A new subgroup of immunoglobulin heavy chain variable region genes for the assessment of clonality in feline B-cell lymphomas. *Vet. Immunol. Immunopathol.* **130,** 59–69 (2009).

92. Lefranc, M. P. Nomenclature of the human immunoglobulin heavy (IGH) genes. *Exp. Clin. Immunogenet.* **18,** 100–116 (2001).

93. Chan, J. K. C. & Kwong, Y. L. Common misdiagnoses in lymphomas and avoidance strategies. *Lancet Oncol.* **11,** 579–588 (2010).

94. Sprouse, MD, J. T. *et al.* T-Cell Clonality Determination Using Polymerase Chain Reaction (PCR) Amplification of the T-Cell Receptor gamma-Chain Gene and Capillary Electrophoresis of Fluorescently Labeled PCR Products. *Am. J. Clin. Pathol.* **113,** 838–850

(2000).

95. van Dongen, J. J. M. *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 concerted action BMH4-CT98-3936. *Leukemia* **17,** 2257–2317 (2003).

96. Lee, S. C., Berg, K. D., Racke, F. K., Griffin, C. A. & Eshleman, J. R. Pseudo-spikes are common in histologically benign lymphoid tissues. *J. Mol. Diagnostics* **2,** 145–152 (2000).

97. Gentilini, F., Turba, M. E. & Forni, M. Retrospective monitoring of minimal residual disease using hairpin-shaped clone specific primers in B-cell lymphoma affected dogs. *Vet. Immunol. Immunopathol.* **153,** 279–288 (2013).

98. Sato, M. *et al.* The prognostic significance of minimal residual disease in the early phases of chemotherapy in dogs with high-grade B-cell lymphoma. *Vet. J.* **195,** 319–324 (2013).

99. Aresu, L. *et al.* Minimal residual disease detection by flow cytometry and PARR in lymph node, peripheral blood and bone marrow, following treatment of dogs with diffuse large B-cell lymphoma. *Vet. J.* **200,** 318–324 (2014).

100. Watson, J. D. & Crick, F. H. C. Genetic Implications of the structure of Deoxyribonucleic Acid. *Nature* **171,** 964–967 (1953).

101. Watson, J. & Crick, F. Molecular structure of nucleic acids. *Nature.* **171,** 737–8 (1953).

102. Dahm, R. Discovering DNA: Friedrich Miescher and the early years of nucleic acid research. *Hum. Genet.* **122,** 565–581 (2008).

103. Henderson, J. T., Benight, A. S. & Hanlon, S. A semi-micromethod for the determination of the extinction coefficients of duplex and single-stranded DNA. *Anal. Biochem.* **201,** 17–29 (1992).

104. Tataurov, A. V., You, Y. & Owczarzy, R. Predicting ultraviolet spectrum of single stranded and double stranded deoxyribonucleic acids. *Biophys. Chem.* **133,** 66–70 (2008).

105. Thermo Scientific, D. NanoDrop Lite: Interpretation of Nucleic Acid 260/280 Ratios. *Protoc. Prod. Manuals* 1 (2012).

106. Iovannisci, D. M., Ha, T. T. & Shaw, G. M. Recovery of genomic DNA from residual frozen archival blood clots suitable for amplification and use in genotyping assays. *Genet. Test.* **10,** 44–9 (2006).

107. Nolan, T., Hands, R. E., Ogunkolade, W. & Bustin, S. A. SPUD: A quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. *Anal. Biochem.* **351,** 308–310 (2006).

108. Deagle, B. E., Eveson, J. P. & Jarman, S. N. Quantification of damage in DNA recovered from highly degraded samples - A case study on DNA in faeces. *Front. Zool.* **3,** 1–10 (2006).

109. Clements, D. N., Wood, S., Carter, S. D. & Ollier, W. E. R. Assessment of the quality and quantity of genomic DNA recovered from canine blood samples by three different extraction methods. *Res. Vet. Sci.* **85,** 74–79 (2008).

110. Kramvis, A., Bukofzer, S. & Kew, M. C. Comparison of hepatitis B virus DNA extractions

from serum by the QIAamp blood kit , GeneReleaser , and the phenol-chloroform Comparison of Hepatitis B Virus DNA Extractions from Serum by the QIAamp Blood Kit , GeneReleaser , and the Phenol-Chloroform Met. *J. Clin. Microbiol.* **34,** 2731–2733 (1996).

111. Aldrich, S. GenElute ™ Mammalian Genomic DNA Miniprep Kit. **63103,** 500 (2010).

112. Technologies, A. Blood Mini Handbook QIAGEN Sample and Assay Technologies. (2012).

113. Roy-Chowdhuri, S. *et al.* Optimizing the DNA yield for molecular analysis from cytologic preparations. *Cancer Cytopathol.* **124,** 254–260 (2016).

114. Killian, J. K. *et al.* Archival fine-needle aspiration cytopathology (FNAC) samples: Untapped resource for clinical molecular profiling. *J. Mol. Diagnostics* **12,** 739–745 (2010).

115. Ausubel, F. M. *et al. Current Protocols in Molecular Biology*. *Molecular Biology* **1,** (2003).

116. Chien, A., Edgar, D. B. & Trela, J. M. Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. *J. Bacteriol.* **127,** 1550–1557 (1976).

117. Technologies, A. Type-it ® Mutation Detect PCR Sample & Assay Technologies QIAGEN Sample and Assay Technologies. (2009).

118. Master, T. & Kit, M. June 2010 TopTaq ™ PCR Handbook TopTaq DNA Polymerase applications without the need for optimization Sample & Assay Technologies QIAGEN Sample and Assay Technologies. (2010).

119. Lonza. GelStar™ Nucleic Acid Gel Stain 10,000X. *Lonza* 8–11 (2014).

120. Motazedian, H., Karamian, M., Noyes, H. A. & Ardehali, S. DNA extraction and amplification of *Leishmania* from archived, Giemsa-stained slides, for the diagnosis of cutaneous leishmaniasis by PCR. *Ann. Trop. Med. Parasitol.* **96,** 31–34 (2002).

121. Oliveira, R. R., Viana, a J. C., Reátegui, a C. E. & Vincentz, M. G. a. Short Communication An efficient method for simultaneous extraction of high-quality RNA and DNA from various plant tissues. *Genet. Mol. Res.* **14,** 18828–38 (2015).

122. Werner, J. A. *et al.* Characterization of Feline Immunoglobulin Heavy Chain Variable Region Genes for the Molecular Diagnosis of B-cell Neoplasia. *Vet. Pathol. Online* **42,** 596–607 (2005).

123. Takanosu, M., Tadika, T. & Kobayashi, T. Heteroduplex polymerase chain reaction is essential for canine receptor rearrangement analysis. *J. Vet. Diagnostic Investig.* **22,** 760–763 (2010).

124. Mochizuki, H. *et al.* Establishment of a novel feline leukemia virus (FeLV)-negative B-cell cell line from a cat with B-cell lymphoma. *Vet. Immunol. Immunopathol.* **140,** 307–311 (2011).

125. Miura, T. *et al.* Structural abnormality and over-expression of the myc gene in feline leukemias. *Int. J. Cancer* **40,** 564–569 (1987).

126. Di Noia, J. M. & Neuberger, M. S. Molecular mechanisms of antibody somatic hypermutation. *Annu. Rev. Biochem.* **76,** 1–22 (2007).

127. Sidney, L. E., Branch, M. J., Dunphy, S. E., Dua, H. S. & Hopkinson, A. Concise review: Evidence for CD34 as a common marker for diverse progenitors. *Stem Cells* **32,** 1380–1389 (2014).

128. Mcsweeney, B. P. A. *et al.* Characterization of Monoclonal Antibodies That Recognize Canine CD34. 1977–1987 (2016).

129. Miettinen, M. & Lasota, J. KIT (CD117): A review on expression in normal and neoplastic tissues, and mutations and their clinicopathologic correlation. *Appl. Immunohistochem. Mol. Morphol.* **13,** 205–220 (2005).

130. Huang, Y.-C. *et al.* CD5-low expression lymphocytes in canine peripheral blood show characteristics of natural killer cells. *J. Leukoc. Biol.* **84,** 1501–1510 (2008).

131. Cobbold, S. & Metcalfe, S. Monoclonal antibodies that define canine homologues of human CD antigens: summary of the First International Canine Leukocyte Antigen Workshop (CLAW). *Tissue antigens* **43,** 137–154 (1994).

132. Moore, P. F. *et al.* Monoclonal antibodies specific for canine CD4 and CD8 define functional T-lymphocyte subsets and high-density expression of CD4 by canine neutrophils. *Tissue Antigens* **40,** 75–85 (1992).

133. Thorarinsdottir, K. *et al.* CD21(-/low) B cells in human blood are memory cells. *Clin. Exp. Immunol.* **185,** 252–262 (2016).

134. Villiers, E., Baines, S., Law, A. M. & Mallows, V. Identification of acute myeloid leukemia in dogs using flow cytometry with myeloperoxidase, MAC387, and a canine neutrophil-specific antibody. *Vet. Clin. Pathol.* **35,** 55–71 (2006).

135. Stokol, T., Schaefer, D. M., Shuman, M., Belcher, N. & Dong, L. Alkaline phosphatase is a useful cytochemical marker for the diagnosis of acute myelomonocytic and monocytic leukemia in the dog. *Vet. Clin. Pathol.* **44,** 79–93 (2015).

136. Moore, P. F., Affolter, V. K. & Vernau, W. Canine hemophagocytic histiocytic sarcoma: a proliferative disorder of CD11d+ macrophages. *Vet. Pathol.* **43,** 632–645 (2006).

137. Yakubenko, V. P. *et al.* The role of integrin alpha D beta2 (CD11d/CD18) in monocyte/macrophage migration. *Exp. Cell Res.* **314,** 2569–2578 (2008).

138. Mcdonough, S. P. & Moore, R. F. Clinical, hematologic, and immunophenotypic characterization of canine large granular lymphocytosis. *Vet. Pathol.* **37,** 637–646 (2000).

139. Langerak, A. W., Szczepanski, T., van der Burg, M., Wolvers-Tettero, I. L. & van Dongen, J. J. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. *Leukemia* **11,** 2192–2199 (1997).

140. Ross, K. S., Haites, N. E. & Kelly, K. F. Repeated freezing and thawing of peripheral blood and DNA in suspension: effects on DNA yield and integrity. *J. Med. Genet.* **27,** 569–70 (1990).

141. Radstrom, P., Knutsson, R., Wolffs, P., Lovenklev, M. & Lofstrom, C. Pre-PCR processing: strategies to generate PCR-compatible samples. *Mol. Biotechnol.* **26,** 133–146 (2004).

142. Groenen, P. J. T. A., Langerak, A. W., van Dongen, J. J. M. & van Krieken, J. H. J. M. Pitfalls

in TCR gene clonality testing: Teaching cases. *J. Hematop.* **1,** 97–109 (2008).

143. Shapiro, G. S., Aviszus, K., Ikle, D. & Wysocki, L. J. Predicting regional mutability in antibody V genes based solely on di- and trinucleotide sequence composition. *J. Immunol.* **163,** 259–268 (1999).

144. Boone, E., Verhaaf, B. & Langerak, A. W. PCR-Based Analysis of Rearranged Immunoglobulin or T-Cell Receptor Genes by GeneScan Analysis or Heteroduplex Analysis for Clonality Assessment in Lymphoma Diagnostics. *Küppers R. Lymphoma. Methods Mol. Biol. (Methods Protoc.* **971,** 65–91 (2013).

145. Dunn-Walters, D., Thiede, C., Alpen, B. & Spencer, J. Somatic hypermutation and B-cell lymphoma. *Philos Trans R Soc L. B Biol Sci* **356,** 73–82 (2001).

146. Nakao, M., Janssen, J. W., Flohr, T. & Bartram, C. R. Rapid and reliable quantification of minimal residual disease in acute lymphoblastic leukemia using rearranged immunoglobulin and T-cell receptor loci by LightCycler technology. *Cancer Res.* **60,** 3281–3289 (2000).

147. Gutzmer, R. *et al.* Detection of clonal T cell receptor γ gene rearrangements in cutaneous T cell lymphoma by LightCycler-polymerase chain reaction. *J. Invest. Dermatol.* **116,** 926–932 (2001).

148. Uemura, A. *et al.* Rapid and high-resolution detection of IgH gene rearrangements using PCR and melting curve analysis. *Int. J. Lab. Hematol.* **29,** 200–207 (2007).

149. Retamales, E. *et al.* Analytical detection of immunoglobulin heavy chain gene rearrangements in gastric lymphoid infiltrates by peak area analysis of the melting curve in the lightcycler system. *J. Mol. Diagnostics* **9,** 351–357 (2007).

150. Langner, K. F. A. *et al.* Detection of clonal antigen receptor gene rearrangement in dogs with lymphoma by real-time polymerase chain reaction and melting curve analysis. *BMC Vet. Res.* **10,** 1 (2014).

151. Schöpper, I. *et al.* Melting curve analysis in canine lymphoma by calculating maximum fluorescence decrease. *Vet. Comp. Oncol.* 1–13 (2016). doi:10.1111/vco.12200

152. Henrich, M., Scheffold, S., Hecht, W. & Reinacher, M. High resolution melting analysis (HRM) for the assessment of clonality in feline B-cell lymphomas. *Vet. Immunol. Immunopathol.* **200,** 59–68 (2018).

153. Keller, S. M. & Moore, P. F. Rearrangement patterns of the canine TCR?? locus in a distinct group of T cell lymphomas. *Vet. Immunol. Immunopathol.* **145,** 350–361 (2012).

154. Davis, M. M. & Bjorkman, P. J. T-cell antigen receptor genes and T-cell recognition. *Nature* **334,** 395–402 (1988).

155. van Dongen, J. J. & Wolvers-Tettero, I. L. Analysis of immunoglobulin and T cell receptor genes. Part II: Possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. *Clin Chim Acta* **198,** 93–174 (1991).

156. Takahashi, T. *et al.* Malignant transformation of T-cell large granular lymphocyte leukemia in a dog. *J. Vet. Med. Sci.* **69,** 677–81 (2007).

157. Bain, B. J., Barnett, D., Linch, D., Matutes, E. & Reilly, J. T. Revised guideline on

immunophenotyping in acute leukaemias and chronic lymphoproliferative disorders. *Clin. Lab. Haematol.* **24,** 1–13 (2002).

158. van den Ancker, W. *et al.* A threshold of 10% for myeloperoxidase by flow cytometry is valid to classify acute leukemia of ambiguous and myeloid origin. *Cytometry B. Clin. Cytom.* **84,** 114–118 (2013).

159. Comazzi, S. *et al.* European canine lymphoma network consensus recommendations for reporting flow cytometry in canine hematopoietic neoplasms. *Cytom. Part B - Clin. Cytom.* **92,** 411–419 (2017).

160. Tasca, S. *et al.* Hematologic abnormalities and flow cytometric immunophenotyping results in dogs with hematopoietic neoplasia: 210 cases (2002-2006). *Vet. Clin. Pathol.* **38,** 2–12 (2009).

161. Bennett, A. L. *et al.* Canine acute leukaemia: 50 cases (1989-2014). *Vet. Comp. Oncol.* 1–14 (2016). doi:10.1111/vco.12251

162. Lagunas-Rangel, F. A., Chávez-Valencia, V., Gómez-Guijosa, M. Á. & Cortes-Penagos, C. Acute myeloid leukemia—genetic alterations and their clinical prognosis. *Int. J. Hematol. Stem Cell Res.* **11,** 329–339 (2017).

163. Nagel, G. *et al.* Epidemiological, genetic, and clinical characterization by age of newly diagnosed acute myeloid leukemia based on an academic population-based registry study (AMLSG BiO). *Ann. Hematol.* **96,** 1993–2003 (2017).

164. Tan, B. T., Seo, K., Warnke, R. A. & Arber, D. A. The frequency of immunoglobulin heavy chain gene and T-cell receptor γ-chain gene rearrangements and Epstein-Barr virus in ALK+and ALK-anaplastic large cell lymphoma and other peripheral T-cell lymphomas. *J. Mol. Diagnostics* **10,** 502–512 (2008).

165. Thériault, C. *et al.* PCR analysis of immunoglobulin heavy chain (IgH) and TcR-gamma chain gene rearrangements in the diagnosis of lymphoproliferative disorders: results of a study of 525 cases. *Mod. Pathol.* **13,** 1269–1279 (2000).

166. Favera, R. D., PELICCI, P.-G. & KNOWLES, D. M. Lymphoid Tumors Displaying Rearrangements of Both Immunoglobulin and T Cell Receptor Genes. *J. Exp. Med.* **162,** 1015–1024 (1985).

167. Hara, J. *et al.* T cell receptor delta gene rearrangements in acute lymphoblastic leukemia. *J Clin Invest* **82,** 1974–1982 (1988).

168. Kay, N. E. Abnormal T-cell subpopulation function in CLL: excessive suppressor (T gamma) and deficient helper (T mu) activity with respect to B-cell proliferation. *Blood* **57,** 418–420 (1981).

169. Lauria, F. *et al.* T-cell functional abnormality in B-chronic lymphocytic leukaemia: evidence of a defect of the T-helper subset. *Br. J. Haematol.* **54,** 277–283 (1983).

170. Zaknoen, S. L. & Kay, N. E. Immunoregulatory cell dysfunction in chronic B-cell leukemias. *Blood Rev.* **4,** 165–174 (1990).

171. Prieto, A. *et al.* Diminished DNA synthesis in T cells from B chronic lymphocytic leukemia after phytohemagglutinin, anti-CD3, and phorbol myristate acetate mitogenic signals. *Exp. Hematol.* **21,** 1563–1569 (1993).

172. Buschle, M. *et al.* Interferon gamma inhibits apoptotic cell death in B cell chronic lymphocytic leukemia. *J. Exp. Med.* **177,** 213–218 (1993).

173. Farace, F. *et al.* T cell repertoire in patients with B chronic lymphocytic leukemia. Evidence for multiple in vivo T cell clonal expansions. *J. Immunol.* **153,** 4281–4290 (1994).

174. Bartik, M. M., Welker, D. & Kay, N. E. Impairments in immune cell function in B cell chronic lymphocytic leukemia. *Semin. Oncol.* **25,** 27–33 (1998).

175. Rezvany, M. R. *et al.* Oligoclonal TCRBV gene usage in B-cell chronic lymphocytic leukemia: major perturbations are preferentially seen within the CD4 T-cell subset. *Blood* **94,** 1063–1069 (1999).

176. Goolsby, C. L., Kuchnio, M., Finn, W. G. & Peterson, L. A. Expansions of clonal and oligoclonal T cells in B-cell chronic lymphocytic leukemia are primarily restricted to the CD3+CD8+ T-cell population. *Commun. Clin. Cytom.* **42,** 188–195 (2000).

177. Wen, T., Mellstedt, H. & Jondal, M. Presence of clonal T cell populations in chronic B lymphocytic leukemia and smoldering myeloma. *J. Exp. Med.* **171,** 659–666 (1990).

178. Moss, P. A. & Gillespie, G. Clonal populations of T-cells in patients with B-cell malignancies. *Leuk. Lymphoma* **27,** 231–238 (1997).

179. Chen, Z. *et al.* Human T cell ? genes are frequently rearranged in B-lineage acute lymphoblastic leukemias but not in chronic B cell proliferations. *J. Exp. Med.* **165,** (1987).

180. Brumpt, C. *et al.* The incidence of clonal T-cell receptor rearrangements in B-cell precursor acute lymphoblastic leukemia varies with age and genotype. *Blood* **96,** 2254–2261 (2000).

181. Vergier, B. *et al.* Combined analysis of T cell receptor gamma and immunoglobulin heavy chain gene rearrangements at the single-cell level in lymphomas with dual genotype. *J. Pathol.* **198,** 171–180 (2002).

182. Gallardo, F. *et al.* Primary cutaneous B-cell lymphoma (marginal zone) with prominent T-cell component and aberrant dual (T and B) genotype; diagnostic usefulness of laser-capture microdissection. *Br. J. Dermatol.* **154,** 162–166 (2006).

183. Bottaro, M., Berti, E., Biondi, A., Migone, N. & Crosti, L. Heteroduplex analysis of T-cell receptor gamma gene rearrangements for diagnosis and monitoring of cutaneous T-cell lymphomas. *Blood* **83,** 3271–3278 (1994).

184. Delfau, M. H., Hance, A. J., Lecossier, D., Vilmer, E. & Grandchamp, B. Restricted diversity of V gamma 9-JP rearrangements in unstimulated human gamma/delta T lymphocytes. *Eur. J. Immunol.* **22,** 2437–2443 (1992).

185. Breit, T. M., Wolvers-Tettero, I. L. & van Dongen, J. J. Unique selection determinant in polyclonal V delta 2-J delta 1 junctional regions of human peripheral gamma delta T lymphocytes. *J. Immunol.* **152,** 2860–2864 (1994).

186. William E. Paul. *Fundamental Immunology*. (LWW, 2012).

187. Sato, H. *et al.* Comparison between Immunohistochemistry and Genetic Clonality Analysis for Cellular Lineage Determination in Feline Lymphomas. *J. Vet. Med. Sci* **73,** 945–947 (2011).

188. Rütgen, B. C. *et al.* Cutaneous T-cell lymphoma - Sézary syndrome in a Boxer. *Vet. Clin. Pathol.* **45,** 172–178 (2016).

189. Gress, V. *et al.* Characterization of the T-cell receptor gamma chain gene rearrangements as an adjunct tool in the diagnosis of T-cell lymphomas in the gastrointestinal tract of cats. *Res. Vet. Sci.* **107,** 261–266 (2016).

190. Nussenzweig, A. & Nussenzweig, M. C. Origin of chromosomal translocations in lymphoid cancer. *Cell* **141,** 27–38 (2010).

191. Weiss, L. M., Warnke, R. A., Sklar, J. & Cleary, M. L. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. *N. Engl. J. Med.* **317,** 1185–1189 (1987).

192. de Boer, C. J., van Krieken, J. H., Schuuring, E. & Kluin, P. M. Bcl-1/cyclin D1 in malignant lymphoma. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* **8 Suppl 2,** 109–117 (1997).

193. Baron, B. W. *et al.* Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5262–6 (1993).

194. Alizadeh, a a *et al.* Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* **403,** 503–11 (2000).

195. Küppers, R. & Dalla-Favera, R. Mechanisms of chromosomal translocations in B cell lymphomas. *Oncogene* **20,** 5580–94 (2001).

196. Aifantis, I., Raetz, E. & Buonamici, S. Molecular pathogenesis of T-cell leukaemia and lymphoma. *Nat. Rev. Immunol.* **8,** 380–390 (2008).

197. Griesser, H. Gene rearrangements and chromosomal translocations in T cell lymphoma--diagnostic applications and their limits. *Virchows Arch.* **426,** 323–338 (1995).

198. Salgado, R. *et al.* Absence of TCR loci chromosomal translocations in cutaneous T-cell lymphomas. *Cancer Genet.* **204,** 405–409 (2011).

199. Jankovic, M. *et al.* Role of the translocation partner in protection against AID-dependent chromosomal translocations. *Proc. Natl. Acad. Sci.* **107,** 187–192 (2010).

200. Shtivelman, E., Henglein, B., Groitl, P., Lipp, M. & Bishop, J. M. Identification of a human transcription unit affected by the variant chromosomal translocations 2;8 and 8;22 of Burkitt lymphoma. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3257–3260 (1989).

201. Teske, E. Canine malignant lymphoma: a review and comparison with human non-Hodgkin's lymphoma. *Vet. Q.* **16,** 209–219 (1994).

202. Hahn, K. A., Richardson, R. C., Hahn, E. A. & Chrisman, C. L. Diagnostic and Prognostic Importance of Chromosomal Aberrations Identified in 61 Dogs with Lymphosarcoma. *Vet. Pathol.* **31,** 528–540 (1994).

203. Thomas, R., Smith, K. C., Ostrander, E. A., Galibert, F. & Breen, M. Chromosome aberrations in canine multicentric lymphomas detected with comparative genomic hybridisation and a panel of single locus probes. *Br. J. Cancer* **89,** 1530–1537 (2003).

204. Winkler, S., Escobar, H. M., Reimann-Berg, N., Bullerdiek, J. & Nolte, I. Cytogenetic investigations in four canine lymphomas. *Anticancer Res.* **25,** 3995–3998 (2005).

205. Devitt, J. J. *et al.* Correlations between numerical chromosomal aberrations in the tumor and peripheral blood in canine lymphoma. *Cytogenet. Genome Res.* **124,** 12–18 (2009).

206. Modiano, J. F. & Breen, M. Shared pathogenesis of human and canine tumors - an inextricable link between cancer and evolution Review Article. **6,** 239–246 (2008).

207. Miura, T. *et al.* Structural abnormality and over-expression of the myc gene in feline leukemias. *Int. J. cancer* **40,** 564–569 (1987).

208. Wu, F. Y., Iijima, K., Tsujimoto, H., Tamura, Y. & Higurashi, M. Chromosomal translocations in two feline T-cell lymphomas. *Leuk. Res.* **19,** 857–860 (1995).

209. Rojko, J. L. *et al.* Feline Lymphomas: Immunological and Cytochemical Characterization. *Cancer Res.* **49,** 345–351 (1989).

210. Chase, M. L. & Armand, P. Minimal residual disease in non-Hodgkin lymphoma – current applications and future directions. *Br. J. Haematol.* **180,** 177–188 (2018).

211. Yamazaki, J. *et al.* Quantitative assessment of minimal residual disease (MRD) in canine lymphoma by using real-time polymerase chain reaction. *Vet. Immunol. Immunopathol.* **126,** 321–331 (2008).

212. Thilakaratne, D. N. *et al.* Clonality and phenotyping of canine lymphomas before chemotherapy and during remission using polymerase chain reaction (PCR) on lymph node cytologic smears and peripheral blood. *Can. Vet. J.* **51,** 79–84 (2010).

213. Sato, M. *et al.* Minimal residual disease in canine lymphoma: An objective marker to assess tumour cell burden in remission. *Vet. J.* **215,** 38–42 (2016).

214. Gentilini, F. *et al.* Real-time quantitative PCR using hairpin-shaped clone-specific primers for minimal residual disease assessment in an animal model of human non-Hodgkin lymphoma. *Mol. Cell. Probes* **24,** 6–14 (2010).

215. Martini, V., Bernardi, S., Marelli, P., Cozzi, M. & Comazzi, S. Flow cytometry for feline lymphoma: a retrospective study regarding pre-analytical factors possibly affecting the quality of samples. *J. Feline Med. Surg.* **20,** 494–501 (2018).

216. Weiss, D. J. Application of flow cytometric techniques to veterinary clinical hematology. *Vet. Clin. Pathol.* **31,** 72–82 (2002).

217. Gengozian, N. Development of monoclonal antibodies to erythroid progenitors in feline bone marrow. *Vet. Immunol. Immunopathol.* **64,** 299–312 (1998).

218. Meister, R. K., Taglinger, K., Haverson, K., Strohminger, N. & Mathes, L. E. Progress in the discovery and definition of monoclonal antibodies for use in feline research. *Vet. Immunol. Immunopathol.* **119,** 38–46 (2007).

219. Araghi, A., Nassiri, S. M., Atyabi, N., Rahbarghazi, R. & Mohammadi, E. Flow cytometric immunophenotyping of feline bone marrow cells and haematopoietic progenitor cells using anti-human antibodies. *J. Feline Med. Surg.* **16,** 265–274 (2014).

220. Barrs, V. & Beatty, J. Feline alimentary lymphoma: 1. Classification, risk factors, clinical signs and non-invasive diagnostics. *J. Feline Med. Surg.* **14,** 182–190 (2012).

Supplementary Information.

Table 1S. Signalment, FC results and clinical presentation of dogs affected by leukaemia. (Chapter 4, Paragraph Three- "Case Description.")

ID	Signalment			Immunophenotype	Clinical signs											
	Age (years)	Sex	Breed		Lethargy	Pyrexia	Anorexia	Exercise intolerance	Lymphadenopathy	Splenomegaly	Mediastinal Mass	Lymphocytosis	Cytopenia	Vomiting/Dhiarroea	Lameness	Cough
1	10	FN	Labrador	AUL						X						
2	10	MN	Labrador	B-ALL		X						X				
4	10	MN	Labrador	T-ALL				X								
5	9	M	Cross Breed	T-ALL	X					with hepatomegaly						
6	7	M	Retriever	T-ALL								X				
7	11	MN	Boxer	AUL	X	X			X						X	
8	11	M	Golden retriever	T-ALL									X			
9	6	M	Labrador	B-ALL							X					
10	2	F	JRT	AUL			X			X						
11	5	MN	Shih tzu	AML								X				
12	7	M	Pointer	T-ALL	X	X										
13	2	ND	Cocker	T-CLL											X	
14	7	FN	Retriever	LGL-ALL									X			
16	10	MN	Labrador	AUL	X											
17	8	M	Weimaraner	biphenotypic					X							X
19	11	FN	Labrador	T-ALL					X	X						
20	2	M	Irish Setter	B-ALL	X	X								X		
21	8	MN	CKCS	biphenotypic	X		X									
22	13	MN	Retriever	T-ALL	X											X

Table 2S. Haematological findings of dogs affected by leukaemia. Labelled in red: values above the reference ranges. Labelled in blue: values below the reference ranges. Labelled in black: values within normal ranges. (Chapter 4, Paragraph 3- "Case Description.").

ID	Diagnosis	Immunophenotype	WBC 6-1 x10^9/L	NEUT 3-11 x10^9/L	LYMP 1-4.8 x10^9/L	MONO 0.2-1.5 x10^9/L	EOS 0.1-1.3 x10^9/L	BASO ≤0.5X10^9/L	ATYPICAL CELLS	RBC 5,5-8,5 x10^{12}/L	Hb 12-18 g/dl	HCT 37-55%	MCV 60-77 fL	MCH 19,5-24,5pg	MCHC 32-37g/dL	RDW 13,2-17,8%	PLT 175-500 x10^9/L	PLT manual count
1	N	AUL	254.19	68.9	30.42		74.29	0.4		4.05	9.3	25.2	62.2	23	37	15.7	nd	nd
2	N	B-ALL	434.51							3.68	9.4	30.6	83.2	25.5	30.7	16.9	nd	nd
4	N	T-ALL	25.9	1.68	23.72	0.13	0.13	0.13		3.35	8.1	23.4	69.9	24.2	34.6	14.8	nd	nd
5	N	T-ALL	71.2	0.71	0.71	0	0	0	69.78	5.25	12.2	41.6	79.2	23.2	29.3			nd
6	N	T-ALL	94.83							3	7.6	22.5	75	25.3	33.8	14	27	consistent
7	N	AUL	18	10.08	3.24	1.26	2.88	0		6.14	13.7	45.8	74.6	22.3	29.9		186	consistent
8	N	T-ALL	116.09	4.64	0	0	0	0	111.4	3.12	7.4	21.2	67.9	23.7	34.9	15.2	9	nd
9	N	B-ALL	57.66	1	54.09	0.4	0.1	0		2.7	5.9	21	79.3	21.8	27.1	16.6	313	consistent
10	N	AUL	14.06							2.33	5.6	19.3	82.8	24	29	14.9	nd	nd
11	N	AML	86.05							4.19	10	34.1	81.4	23.9	29.3	20	268	consistent
12	N	T-ALL	279.85							3.15	7.8	24.9	79	24.8	31.3	16	200	consistent
13	N	T-CLL	15.79	4.64	10.04	1.06	0.04	0.01		3.36	7.9	24.9	74.1	23.5	31.7	17.8	74	consistent
14	N	LGL-ALL	77.92							3.99	9.8	31.5	78.9	24.6	31.1	15.1	nd	nd
16	N	AUL	13.40	1.072	11.79	0.4	0.13	0		2.27	5.1	14.8	64.9		34.4		150	consistent
17	N	biphenotypic	75.9	0.17	38.63	37.09		0		1.69	3.8	10.8	63.9	22.5	35.2	15.6	357	consistent
19	N	T-ALL	11.04							1.15	3.6	12.6	109.6	31.3	26.6		nd	nd
20	N	B-ALL	54.89							6.17	15.9	47	77.6	25.8	33.2		nd	nd
21	N	biphenotypic	11.52	1.1	9.2	0.91	0.23	0.01		3.57	9.5	24.5	68.6	26.6	38.7	16	11	consistent
22	N	T-ALL	28	2.5	23	2.2	0	0		4.59	11.4	35	76.3	24.8	32.6		288	consistent

Table 3S. Signalment and haematological findings of dogs affected by reactive leukocytosis. Labelled in red: values above the reference ranges. Labelled in blue: values below the reference ranges. Labelled in black: values within normal ranges. (Chapter Four, Paragraph Three- "Case Description.")

ID	Diagnosis	Signalment			WBC	NEUT	LYMP	MONO	EOS	BASO	ATYPICAL CELLS	RBC	Hb	HCT	MCV	MCH	MCHC	RDW	PLT	PLT manual count
		Age (years)	Sex	Breed	6-1 x10^9/L	3-11 x10^9/L	1-4.8 x10^9/L	0.2-1.5x10^9/L	0.1-1.3 x10^9/L	≤0.5 x10^9/L		5.5-8.5 x10^{12}/L	12-18 g/dl	37-55%	60-77 fL	19.5-24.5pg	32-37g/dL	13.2-17.8 %	175-500 x 10^9/L	
A	R	9	FN	Rodhesian Ridgeback	14.7	6.91	7	0.47	0.3			9.61	18.9	60.3	62.7	22.8	36.3	19.8	17	consistent
B	R	10	FN	Cocker Spaniel	60.67	49.75	9.1	0.61	1.21	0		3.31	7.4	25.8	77.9	22.4	28.7	21.9	100	consistent
C	R	1	F	Shnauzer	16.06	8.69	6.4	0.35	0.42	0.17		6.42	12.5	36.4	56.7	19.5	34.3	16.4	54	nd
D	R	3	FN	Cocker Spaniel	31.45	21.39	7.2	2.83	0	0		6.88	15.1	42	61	21.9	36	14.4	227	consistent
E	R	1	M	Basset Hound	19.21	12.16	5.2	0.77	1.1	0.02		6.06	15.1	43.6	71.9	24.9	34.6	14	44	consistent
F	R	2	FN	Flat Coated Retriever	67.16	48.84	8.4	8.4	1.53			3.87	9.5	29.8	77	24.5	31.9	19.4	252	consistent
G	R	Nd	M	Lhasa apso	18.59	9.92	6.5	1.34	0.82	0.04		6.07	11.4	33.6	55.4	18.8	33.9	18.7		nd
H	R	5	MN	Labradoodle	11.09	4.28	5.6	0.34	0.83	0.02		6.71	16.4	46.3	69	24.4	35.4	15.1	26	nd
I	R	2	MN	Pug	34.56	25.86	5.9	2.31	0.42	0.03		6.93	13.8	37.8	54.5	19.9	36.5	18	211	consistent
J	R	8months	FN	Cocker Spaniel	20.09	11.62	6.9	0.81	0.72	0.09		4.97	11.6	33.3	67	23.3	34.8	14.7	9	normal

Table 4S. Complete extraction data from the feline cases selected. **N-T**, Neoplasia of T cell; **N-B**, neoplasia of B cells; R, reactive process; **ND,** not a definitive diagnosis (see the text for description); **LN**, lymphnode; **DSH**, Domestic Short Hair; **DLH**, Domestic Long Hair; **GROUP 1**, samples fulfilling the optimal requirements for gDNA; **GROUP 2**,samples which showed good quality characteristics, even with low concentrations of DNA, included in the study; **EXCLUDED**, samples excluded from the study due to low quality gDNA. (Chapterf Five, Paraghraph Three, "Extraction Data").

Sample ID	Immunophenotype	Age (years)	Breed	Localisation	N. of slides used	DNA concentration (ng/µL)	$A_{260/280}$ ratio	$A_{260/230}$ ratio	
4	**N-T** (CD5+/CD4-/CD8-/CD21-)	n/a	n/a	LN	1	33.37	1.81	1.785	GROUP 1
10	**N-B** (CD5-/CD4-/CD8-/CD21+)	10	DSH	Blood	1	44.72	1.81	2.13	
15	**N-T** (CD5+/CD4-/CD8+/CD21-)	14	DSH	Renal mass	5	79.4	1.82	1.9	
16	**R**	11	DSH	LN	2	103.81	1,83	2.06	
17	**R**	14	DLH	LN	3	119.06	1.87	2.22	
18	**N-B** (CD5-/CD4-/CD8-/CD21+)	5	DSH	Prescapular LN	2	81.99	1.93	2.65	
25s	**R**	8	DSH	Spleen	4	153.88	1.94	1.86	
25L				Mesenteric LN	2	59.49	1.91	1.32	
29	**ND**	5	DSH	LN	4	48.76	1.8	1.43	
32	**R**	8	Burmilla	Right submandibular LN	2	34.39	1.81	2.12	
33	**ND**	12	DLH	Jejunal LN	5	140.39	1.87	2	
34	**N-T** (CD5+/CD4-/CD8-/CD21-)	2	DSH	Mass on caudal dorsum	4	45.84	1.85	1.73	
36	**N-B** (CD5-/CD4-/CD8-/CD21+)	11	DSH	LN	3	500	1.96	2.11	
3	**N-T** (CD5+/CD4+/CD8-/CD21-)	12	DSH	Blood	1	13.955	1.775	0.675	GROUP 2
5	**N-B** (CD5-/CD4-/CD8-/CD21+)	10	Birman	Jejunal LN	1	22.23	1.915	1.66	
7	**N-T** (CD5+/CD4+/CD8-/CD21-)	13	DSH	Blood	2	14.355	1.88	1.615	
12	**R**	1	DSH	LN	3	8.07	1.97	1.73	
22	**N-B** (CD5-/CD4-/CD8-/CD21+)	13	DSH	Colonic mass	2	22.305	1.88	1.39	
24	**N-B** (CD5-/CD4-/CD8-/CD21+)	12	DSH	Intestinal mass	1	12.12	1.94	2.19	
27	**N-T** (CD5+/CD4+/CD8-/CD21-)	13	DSH	Blood	2	22.95	1.78	1.44	
28	**ND**	n/a		Abdominal LN	3	21.055	1.9	1.73	
31	**N-T** (CD5+/CD4+/CD8-/CD21-)	n/a	DSH	Abdominal LN	2	19.58	1.81	1.62	
1 si	**N-T** (CD5+/CD4+/CD8-/CD21-)	16	DSH	Small Intestine	1	7.115	2.11	1.19	EXCLUDED
1l	**R**			Liver	2	6.65	2.075	0.625	
2	**ND** (all markers negative)	7	Siamese	Blood	1	2.965	3.155	0.44	
6	**R**	1	DSH	Abdominal LN	2	6.735	1.785	1	
8	**R**	2	DSH	Blood	2	3.37	2.16	0.865	
9	**R**	15	British Blue	Spleen	2	9.42	1.58	1.29	
11	**R**	12	DSH	LN	1	2.56	1.84	0.38	
13	**R**	2	DSH	Mesenteric LN	2	6.455	1.54	1.42	
14	**N-T** (CD5+/CD4+/CD8-/CD21-)	9	DSH	Blood	3	2.485	1.47	0.35	
19	**N-T** (CD5+/CD4+/CD8-/CD21-)	18	Persian X	Blood	1	8.8	2.33	21.84	
20	**N-T** (CD5+/CD4-/CD8+/CD21-)	n/a	DSH	Blood	1	3.385	2.34	0.345	
21	**N-T** (CD5+/CD4+/CD8-/CD21-)	14	SDSH	Blood	2	4.29	2.09	0.795	
23	**R**	1	DSH	jejunal LN	2	6.93	2.075	1.1	
26	**N-T** (CD5+/CD4+/CD8-/CD21-)	15	DSH	Jejunal LN	3	12.525	2.205	0.615	
30	**R**	6	Savannah	Jejunal LN	1	7.88	1.415	0.635	
35	**R**	8	DSH	Mediastinal mass	1	6.855	2.04	0.315	
37	**R**	12	DSH	Mesenteric LN	2	10.855	2.1	2.185	