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First time application of a clonality assay in a large cohort of non- domestic felines

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I am deeply grateful for all the people who have made it possible for me to stand at this point in my life. I want to name just a few of them, although there are and will be so much more.

First of all, I want to thank my supervisor Dr. Sabine E. Hammer for her support and guidance through the process of this manuscript.

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Nevertheless, I would not be here without my family. I would like to thank my parents and my sister, who supported me during my studies and always had my back no matter what.

And last but not least, I am so grateful for all the people who made the years of my studies so special and unforgettable.

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1 Introduction

Lymphoma, the most common tumor in domestic cats, is increasingly diagnosed in non-domestic felines. Although the exact prevalence of lymphoma in different feline species is unknown, the occurrence of malignant lymphoma in captive African lions (*Panthera leo*), is well- reported (Harrison et al., 2010). The African lion suffers predominantly from T-cell lymphoma. The main clinical signs include weight loss, splenomegaly and spleen, liver and regional lymph node involvement. Although there is a confirmed correlation between lymphoma and feline leukemia virus (FeLV) as well as feline immunodeficiency virus (FIV) in domestic cats, there is no such association in lions.

Apart from lions, lymphoma cases have been reported in other feral feline species including cheetahs (*Acinonyx jubatus*) and jaguars (*Panthera onca*) (Marker et al., 2003; Keresztes et al., 2017).

Keresztes et al. (2017) reported a non- regenerative immune- mediated anemia associated with a diffuse large B-cell lymphoma in a captive jaguar (*Panthera onca*) (Keresztes et al., 2017). The animal presented with anorexia and hemostasis dysfunction prior to the diagnosis. The primary sites of neoplastic growth were the spleen, the liver and, in contrary to the reported cases of T-cell lymphomas, peripheral lymph nodes.

As in African lions, a multicentric T-cell lymphoma has been reported in a captive Namibian cheetah (*Acinonyx jubatus*). In this case, which was associated with feline leukemia virus infection, the liver, spleen, lymph nodes and other organs proved to be infiltrated with neoplastic T- lymphocytes (Marker et al., 2003).

In 1991, a captive lion tested positive for feline immunodeficiency virus (FIV), after showing typical clinical signs for a lentivirus infection (Poli et al., 1995). In addition to other abnormalities, lymphoma was detected and classified as diffuse small lymphocytic lymphoma (DSLL).

Basic diagnostics in the above cases included common methods such as physical examination, total blood count, blood chemistry and further diagnostics like ultrasound, fine needle aspiration, biopsies and radiography. The gold standard for the diagnosis of feline lymphoma is the histopathological examination of tissue samples.

As a useful adjunct diagnostic tool, the PCR-based lymphocyte clonality assay has been tested, improved and established (van Dongen et al., 2003). The method is based on the

different characteristics of reactive and malignant lymphocytes. Neoplastic B- and T-cells, originating from one single transformed cell, can be distinguished from benign lymphocytes by their antigen receptor gene rearrangements (Keller et al., 2016). The different assemblies of the immunoglobulin heavy chain (IGH) variable region and the T-cell receptor gamma chain (TRG) originate by random recombination of variable (V), diversity (D), and joining (J) regions (Jung et al., 2006). This entails that all tumorous cells contain a unique clonal IGH or TRG gene rearrangement (Keller et al., 2016). To interpret the clonality of lymphocyte populations, the PCR-products are assessed using multicapillary gel electrophoresis, a Genescan device. The clonality patterns illustrate the difference between reactive and neoplastic lymphocytes and could therefore serve as an adjunct tool in the diagnosis of lymphoma in non-domestic felines. Therefore, the aim of this study was to test the applicability of the primer sets used to assess clonality in domestic cats, for wild, namely non-domestic felines. In this study, clonality could be detected in eight non-domestic felines, including three lymphoma cases being confirmed by histopathology. These results point towards the specific usability of domestic feline-specific IGH-VDJ or TRG-VJ primers in non-domestic feline species.

2 Material and methods

2.1 Case Selection and Origin of Sample Material

Between 2010 and February 2020, spleen and lymph node samples were taken from 41 exotic felids during routine post- mortem examinations at the Research Institute of Wildlife Ecology (Vetmeduni Vienna). After necropsy, the specimens were stored in small containers and frozen at – 80 ˚C. Included in this study are eight different non- domestic feline species: *European wildcat (Felis silvestris), Eurasian lynx (Lynx lynx), tiger (Panthera tigris), jaguar (Panthera* onca), African lion (*Panthera leo*), leopard (*Panthera pardus*), cougar (*Puma concolor*) and cheetah (*Acinonyx jubatus*). Thirty-three of these animals lived in captivity and seven originated from the wild (Tab. 1, Suppl. Tab. S1).

Tab. 1. Condensed case history of 41 non- domestic feline individuals (equal to 45 gDNA samples) analyzed in this study.

Case Number	Sample Number	Common Name	Age	Origin	Sampling date	Localization	Lymphoma[1]	Cause of death
1	1	European wildcat	1 year	zoo collection	2010	lymph node	No	suffocation
2	2	European wildcat	6 years	zoo collection	2015	lymph node	No	culling
4	3	European wildcat	N/d	wild	2016	spleen	No	FIP
3	4	European wildcat	10 years	zoo collection	2016	spleen	No	renal failure
5	5	European wildcat	juvenile	zoo collection	2020	lymph node	No	euthanasia
	6					spleen	No	
6	7	European wildcat	adult	zoo collection	2020	lymph node	No	FIP
	8					spleen	No	
7	9	European wildcat	juvenile	zoo collection	2020	lymph node	No	FIP
	10					spleen	No	
8	11	Eurasian lynx	N/d	N/d	N/d	lymph node	No	N/d
9	12	Eurasian lynx	juvenile	wild	2012	lymph node	No	trauma, hit by car
10	13	Eurasian lynx	N/d	wild	2013	lymph node	No	cachexia

11	14	Eurasian lynx	< 1 year	wild	2014	lymph node	No	trauma
12	15	Eurasian lynx	2.5 months	wild	2015	lymph node	No	trauma
13	16	Eurasian lynx	20.5 years	zoo collection	2016	lymph node	No	multiple organ failure
14	17	Eurasian lynx	4 years	zoo collection	2016	spleen	No	Not clear
15	18	Eurasian lynx	2 years	wild (national park)	2016	lymph node	No	Not clear
16	19	Tiger	15 years	zoo collection	2010	lymph node	Yes	euthanasia
17	20	Tiger	9 years	zoo collection	2017	lymph node	Yes	lymphoma
18	21	Tiger	juvenile	zoo collection	2019	spleen	No	septicemia
19	22	Tiger	juvenile	zoo collection	2019	spleen	No	euthanasia
20	23	Jaguar	17 years	zoo collection	2010	lymph node	No	multiple organ failure
21	24	Jaguar	22 years	zoo collection	2015	lymph node	No	euthanasia
22	25	Jaguar	22 years	zoo collection	2015	lymph node	No	euthanasia
23	26	Lion	18 years	zoo collection	2017	lymph node	No	euthanasia
24	27	Lion	5 years	zoo collection	2018	lymph node	No	euthanasia
25	28	Lion	neonate	zoo collection	2019	spleen	No	dystocia
	29					lymph node	No	
26	30	Leopard	21.5 years	zoo collection	2015	lymph node	No	euthanasia

27	31	Cougar	1 day	zoo collection	2010	lymph node	No	still birth
28	32	Cougar	1 day	zoo collection	2010	lymph node	No	dystocia
29	33	Cougar	7 years	zoo collection	2017	lymph node	No	septicemia
30	34	Cheetah	9 years	zoo collection	2010	lymph node	No	drowning
31	35	Cheetah	15 years	zoo collection	2010	lymph node	No	circulatory collapse
32	36	Cheetah	3 days	zoo collection	2010	spleen	No	septicemia
33	37	Cheetah	10 years	zoo collection	2011	spleen	Yes	multiple organ failure
34	38	Cheetah	neonate	zoo collection	2014	spleen	No	still birth
35	39	Cheetah	13 years	zoo collection	2015	spleen	No	euthanasia
36	40	Cheetah	6 months	zoo collection	2015	lymph node	No	drowning
37	41	Cheetah	8 years	zoo collection	2015	lymph node	No	euthanasia
38	42	Cheetah	2 years	zoo collection	2016	lymph node	No	euthanasia
39	43	Cheetah	14 years	zoo collection	2017	lymph node	No	malabsorption syndrome
40	44	Cheetah	N/d	wild	2017	spleen	No	culling
41	45	Cheetah	9 years	zoo collection	2019	spleen	No	euthanasia

N/d, not determined; [1]based on the histopathological examination at the Research Institute of Wildlife Ecology (Vetmeduni Vienna)

2.2 Histopathological examination

As part of the dissection, the organs were routinely sampled and put in 7 % neutral buffered formalin for fixation. Slices of 4 µm thickness were cut from the different tissues and placed onto microscope slides. The slides were stained with hematoxylin and eosin (HE stain; Merck KGaA, Darmstadt, Germany) and examined under the microscope.

2.3 DNA extraction and gDNA quality control

Total genomic DNA (gDNA) was extracted by using the E.Z.N.A Tissue DNA Kit (Omega Biotech, Norcross, Georgia) following the manufacturer's instructions. Between 30 and 60 mg of tissue were minced and further processed as follows. The minced tissue was transferred to a 1.5 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany) and 400 µL of TL buffer were added. After the addition of 25 µL of OB Protease Solution, the mixture was thoroughly vortexed. The tissue-buffer solution was incubated overnight at 55 °C in a shaking water bath, to enable complete tissue lysis. For RNA digestion, 4 µL RNase A (100 mg/mL) was added and the samples were incubated for 2 minutes (min.) at room temperature. For subsequent cell lysis, 420 µL BL buffer were added, followed by another incubation step at 70 °C for 10 min. Next, the adjusted amount of 420 µL of 100 % ethanol was added and the mixture thoroughly vortexed. The whole sample was transferred to an assembled HiBind®DNA Mini Column and centrifuged at 13,200 rpm (Microcentrifuge, Eppendorf AG) for 1 min. After discarding the flow-through, 500 µL of HBC Buffer were added followed by a centrifuge step at 13,200 rpm for 1 min. Again, the flow-through was discarded and two washing steps were performed by adding 700 µL DNA Wash Buffer followed by a centrifuge step at 13,200 rpm for 1 min. To dry the mini column, the empty HiBind®DNA Mini Column assembly was centrifuged at 13,200 rpm for 2 min. The mini column was transferred to another 1.5 mL Eppendorf tube (Eppendorf AG). Finally, gDNA was eluted from the mini column by adding 100-200 µL pre-heated (70 °C) Elution Buffer and the entire sample was left at room temperature for 2 min. and centrifuged at 10,000 rpm for 2 min. This step was repeated by reloading the flow-through to the mini column to perform a second elution step. The concentration as well as the quality of the extracted gDNA were assessed with the Nano Drop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) in pedestal mode. Per gDNA sample, at least two measurements were performed, making sure that the two values would not differ more than 5 ng/µL. The threshold was set to 30 ng/µL with desired 260/280 ratios of 1.8-2.0 and 260/230 ratios of above or equal two (2.0-2.2).

To evaluate the suitability of the gDNA for the clonality assay, a 189 bp fragment of the feline androgen receptor gene (fAR) was PCR-amplified for each sample using the following primers: forward primer 5'-CACAATGCCGCTACGGGGACCT-3' (Mochizuki et al., 2012) and reverse primer 5'-AGGGGGTCACAGACCCTGACTCG-3' (Mochizuki, pers. com.). The PCR reaction contained 2x 4x TopTaq AllTaq PCR Master Mix (Qiagen GmbH, Hilden, Germany), 1× CoralLoad® (Qiagen), 10-40 pM of each fAR primer (Eurofins Genomics, Ebersberg, Germany) and 150 ng of eluted gDNA (>30 ng/µL; Suppl. Tab. S2) as template brought up to 12.5 µL with molecular biology grade water (Qiagen). Additionally, the feline lymphoma cell lines MS4 and FT-1 (Mochizuki et al., 2012; Mochizuki et al., 2011) were used as positive controls and the size of the PCR product was evaluated using standard agarose gel electrophoresis.

2.4 PCR protocol for the Clonality Testing

For the amplification of the IGH-VDJ and TRG-VJ gene rearrangements, specific primer sets designed by Mochizuki and co-workers and synthesized by Eurofins Genomics were used (Mochizuki et al., 2012; Mochizuki et al., 2011). In total, six primer sets were applied to target complete IGH-VDJ rearrangements, and a multiplex primer set was used to target TRG specific genes (primer set B in Hammer et al., 2017). IGH-VDJ and TRG-VJ gene rearrangements were amplified as previously described for primer set B (Hammer et al., 2017). After PCR, 10 µL of DNA Dilution Buffer (Qiagen) were added to each PCR reaction and size separated using the QIAxcel Advanced System capillary electrophoresis analyzer with the QIAxcel DNA High Resolution Kit and the QX Alignment Marker 15 bp/1000 bp (Qiagen). The presence and size of obtained PCR products was accurately determined using QIAxcel ScreenGel Software (Qiagen) (Hammer et al., 2017; Gress et al., 2016).

2.5 Interpretation of clonality patterns

The clonality patterns were interpreted using guidelines for clonality testing in veterinary medicine (Keller et al., 2016) and the EuroConality/BIOMED-2 guidelines for clonality testing in human medicine (Langerak et al., 2012). Identical PCR triplicates verified the reproducibility of the clonality patterns for every sample and PCR- reactions not following this rule were defined as pseudoclonal. The patterns were categorized according to their peak morphology. Monoclonal peaks were defined as being at least double the height of the background, whereas polyclonality was characterized by multiple peaks being organized in a bell-shaped curve.

Definite single peaks surrounded by a polyclonal setting were defined as monoclonal with a polyclonal background (Hammer et al., 2017; Gress et al., 2016). More than one clone in a lesion resulting in at least two peaks would indicate oligoclonality (Keller et al., 2016).

Diagnostic sensitivity, diagnostic specificity and accuracy of the test for this non-domestic feline cohort was calculated based on the histopathological diagnosis as a gold standard defining true positive, true negative, false negative and false positive clonality results (Stockham and Scott, 2008).

3 Results and discussion

3.1 Impact of gDNA quality on the results of the PCR- based clonality assay

The 45 gDNA samples tested originated from seven wildcats, eight lynx, four tigers, three jaguars, three lions, one leopard, three cougars and twelve cheetahs with ages ranging from neonatal to 22 years of age. For nine animals, the exact ages where unknown. Thirty- three animals belonged to different zoo collections and seven animals originated from the wild.
Detailed information about the studied animals including full case history and clinical signs are given in Tab. 1 and Suppl. Tab. S1. For three European wildcats (case no. 5, 6 and 7) and one lion (case no. 25), the gDNAs derived from two different tissues, lymph node and spleen.
Genomic DNA concentrations ranged from 34.3 to 1166.1 ng/µL with a mean value of 381.05 ng/µL (Suppl. Tab. S2). The 260/280 ratio presented with a maximum of 2.16, a minimum of 1.81 and a mean value of 1.91. The results of the 260/230 ratio showed a maximum of 2.63, a minimum of 1.89, next to a mean value of 2.25 (Suppl. Tab. S2). In total, 45 tissue samples obtained from 41 animals were subjected to the amplification of the 189 bp fragment of the feline androgen receptor gene (fAR), serving as internal PCR control. Despite 38 positive samples, seven out of the twelve cheetah gDNAs failed in the fAR PCR because of sequence ambiguities at the fAR forward primer-binding site in the cheetah genomes. The gDNA concentrations of the fAR-negative cheetah samples ranged from 98.10 to 787.75 ng/µL, the 260/30 ratios lay within 1.86 and 1.92, and the 260/30 ratios ranged between 2.01 and 2.32, hence complying to the recommended gDNA quality standards as postulated by Hammer and co-authors in 2017 (Hammer et al., 2017). Despite the negative fAR PCR results, we decided to include all twelve cheetahs in this study to ensure we did not lose valuable scientific information about the cheetah cases. Hence, all 45 non-domestic feline samples (equal to 41 non-domestic feline individuals) were assayed for their IGH-VDJ and TRG-V-J gene rearrangements by clonality testing (Suppl. Tab. S2).

3.2 Consistency of clonality patterns with histopathological evidence

After PCR-based lymphocyte clonality assay and subsequent Genescan analysis of the PCR products, eight samples from eight cases [case 7 (localization spleen), 10, 16, 17, 20, 21, 24, 33)] showed clonal results, two of them of T-cell and four of them of B-cell clonality and another two of B- and T-cell monoclonality (Fig. 1, Tab. 2, Suppl. Tab. S3). Three of these samples (two tigers and one cheetah, case no. 16, 17 and 33, sample no. 19, 20 and 37) are confirmed

lymphoma cases, based on histopathology and hence showing a true positive result (Suppl. Tab. S1). All these animals were kept in zoos and were between nine and fifteen years old. Lymph nodes were used as sample material for two of them (two tigers, case no. 16 and 17, sample no. 19 and 20) and spleen tissue for the third one (cheetah, case no. 33, sample no. 37) (Tab. 2). At the dissection, a ten-year-old, female cheetah (case no. 33, sample no. 37) showed, in addition to a lymphoma in the spleen, chronic alterations in the kidneys and the stomach, as well as age- related changes of liver, heart, kidneys and brain. The PCR- based clonality assay confirmed a T-cell lymphoma with a distinct monoclonal peak, as shown in Fig. 2. Furthermore, the method could verify a T-cell lymphoma of a female nine-year-old tiger (case no. 17, sample no. 20) who presented with a thymus lymphoma associated with metastases in the intestines (Fig. 2). The third case, a male fifteen-year-old tiger (case no. 16, sample no. 19) was euthanized after a history of inappetence, hind limb weakness, polydipsia and kidney failure. The following pathological dissection revealed an intestinal lymphoma and degenerative changes in multiple organs. The clonality assay resulted in a B and T-cell clonality, supporting the histological findings from the dissection (Fig. 2). The lymphocytes derived from one neoplastic cell result in a monoclonal peak, as they have a homogenous antigen gene rearrangement.

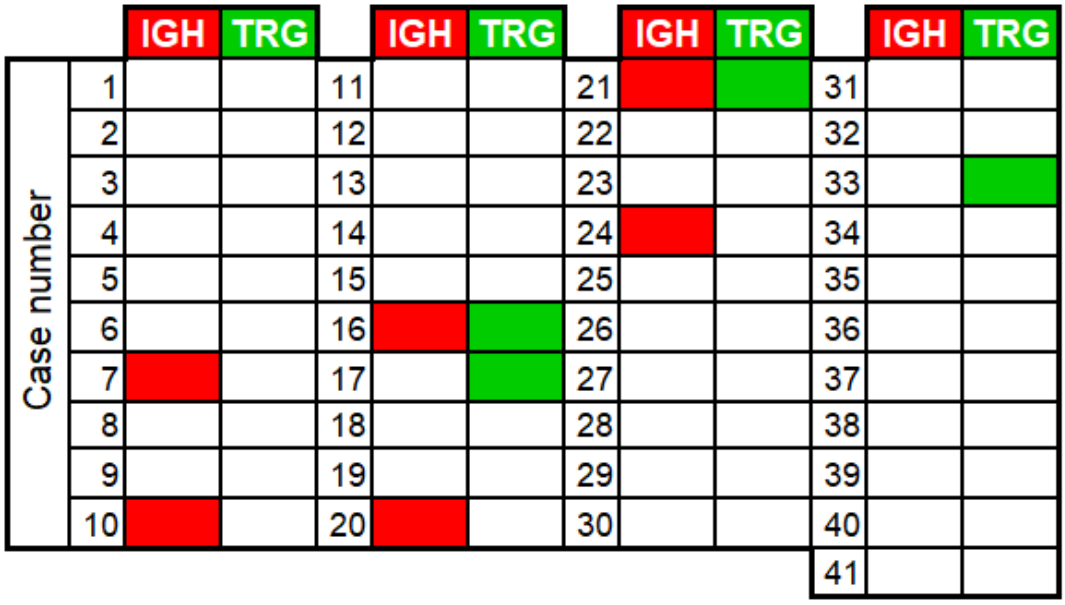

Fig. 1. This heat map summarizes the occurrence of B- and T-cell clonality in 41 non-domestic feline cases as detected with IGH-VDJ and TRG-VJ specific primer sets. Cases 16 and 21 showed B- and T-cell clonality. Only B-cell clonality was found for cases 7, 10, 20 and 24; whereas cases 17 and 33 were positive for T-cell clonality.

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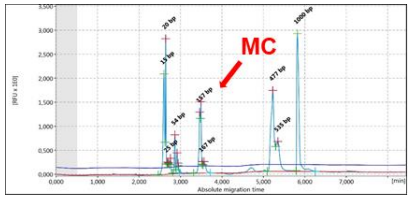
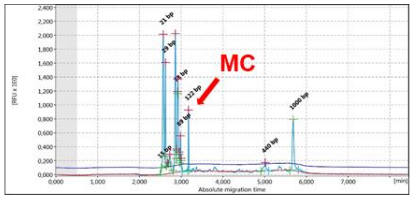
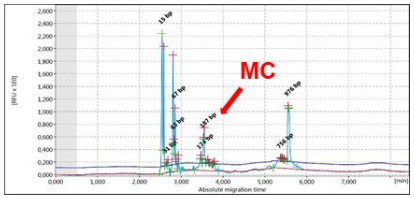
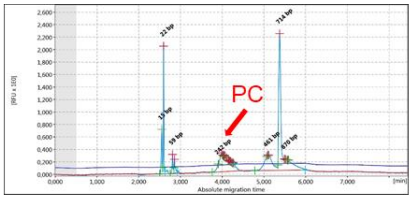
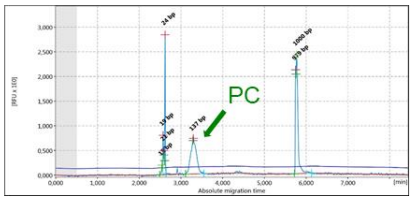


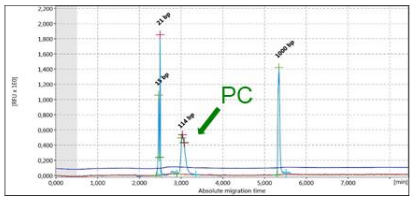


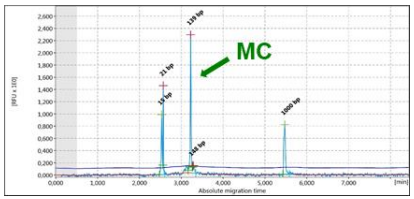


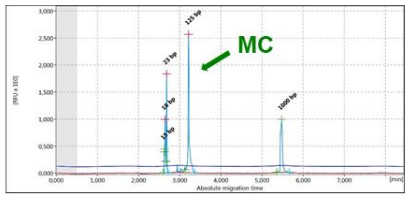


TRG



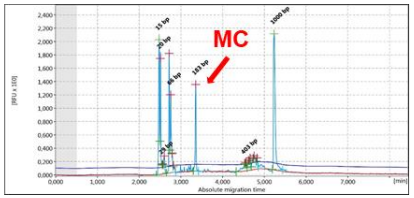


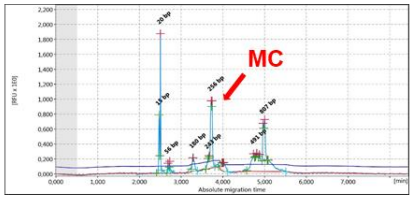


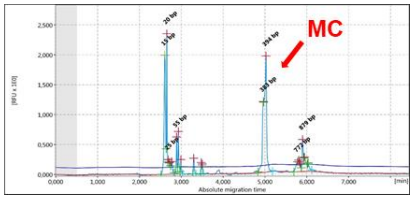


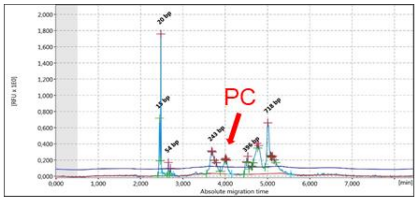
Case #7 Case #10 Case #16 Case #17

IGH

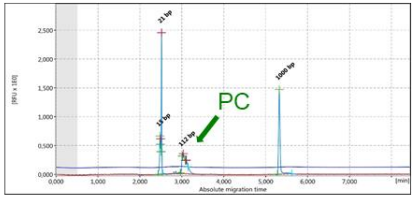


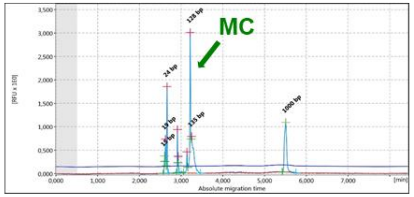


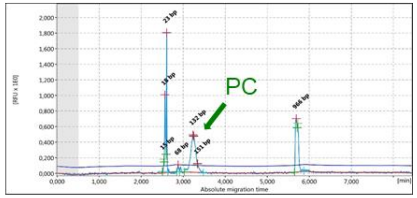


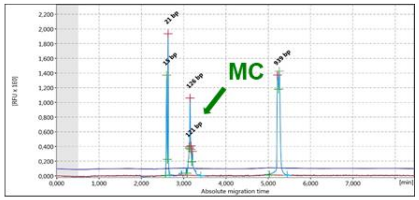


TRG









Case #20 Case #21 Case #24 Case #33

Fig. 2. Representative clonality patterns (electropherograms) of the eight positive non- domestic feline cases 7, 10, 16, 17, 20, 21, 24 and 33 for the IGH-VDJ and TRG-VJ primer sets. Cases 16 and 21 show a monoclonal population in both reaction setups. Cases 7, 10, 20 and 24 have a monoclonal peak in reaction setup IGH and a polyclonal distribution in primer mix TRG. Cases 17 and 33 revealed a monoclonal peak in the TRG-specific reaction mix and a polyclonal distribution with IGH-specific primers. The expected amplicons ranged from ~100 to ~400 bp and primer dimers ranged from 60 to 70 bp. MC, monoclonal; PC, polyclonal; RFU, relative fluorescent units

Tab. 2. Summarizing results of 41 non- domestic feline individuals (equal to 45 gDNA samples) analyzed in this study.

Case Number	Sample Number	Common Name	Localization	Clonality	Lymphoma[1]
1	1	European wildcat	lymph node	N/d	No
2	2	European wildcat	lymph node	N/d	No
3	3	European wildcat	spleen	N/d	No
4	4	European wildcat	spleen	N/d	No
5	5	European wildcat	lymph node	N/d	No
	6		spleen	N/d	No
6	7	European wildcat	lymph node	N/d	No
	8		spleen	N/d	No
7	9	European wildcat	lymph node	N/d	No
	10		spleen	B-cell	No
8	11	Eurasian lynx	lymph node	N/d	No
9	12	Eurasian lynx	lymph node	N/d	No
10	13	Eurasian lynx	lymph node	B-cell	No
11	14	Eurasian lynx	lymph node	N/d	No

12	15	Eurasian lynx	lymph node	N/d	No
13	16	Eurasian lynx	lymph node	N/d	No
14	17	Eurasian lynx	spleen	N/d	No
15	18	Eurasian lynx	lymph node	N/d	No
16	19	Tiger	lymph node	B-and T-cell	Yes
17	20	Tiger	lymph node	T-cell	Yes
18	21	Tiger	spleen	N/d	No
19	22	Tiger	spleen	N/d	No
20	23	Jaguar	lymph node	B-cell	No
21	24	Jaguar	lymph node	B- and T- cell	No
22	25	Jaguar	lymph node	N/d	No
23	26	Lion	lymph node	N/d	No
24	27	Lion	lymph node	B-cell	No
25	28	Lion	spleen	N/d	No
	29		lymph node	N/d	No
26	30	Leopard	lymph node	N/d	No
27	31	Cougar	lymph node	N/d	No

28	32	Cougar	lymph node	N/d	No
29	33	Cougar	lymph node	N/d	No
30	34	Cheetah	lymph node	N/d	No
31	35	Cheetah	lymph node	N/d	No
32	36	Cheetah	spleen	N/d	No
33	37	Cheetah	spleen	T-cell	Yes
34	38	Cheetah	spleen	N/d	No
35	39	Cheetah	spleen	N/d	No
36	40	Cheetah	lymph node	N/d	No
37	41	Cheetah	lymph node	N/d	No
38	42	Cheetah	lymph node	N/d	No
39	43	Cheetah	lymph node	N/d	No
40	44	Cheetah	spleen	N/d	No
41	45	Cheetah	spleen	N/d	No

N/d, not detected; [1]based on the histopathological examination at the Research Institute of Wildlife Ecology (Vetmeduni Vienna).

3.3 False positive clonality patterns as compared with the histopathological gold standard

Five samples showed no evidence of neoplastic lymphocytes at histopathology, but monoclonality of B- and/or T-cell antigen receptor gene rearrangements representing false positive clonality results (Fig. 1, Tab. 2, Suppl. Tab. S3). A female jaguar, 22 years of age (case no. 21, sample no. 24), presented with a mastocytoma with metastases and potential hypereosinophilic syndrome together with other degenerative changes (Suppl. Tab. S1). Contrary to the findings of the pathology, the outcome of the clonality assay was positive for V3F3 and TRG-J2 primers. The most significant findings of another jaguar, male and 17 years old (case no. 20, sample no. 23), included multiple myelolipomas in the spleen, lymphadenitis and chronic inflammation signs in several organs, whereas the clonality assay revealed a monoclonal peak for the primer targeting IGH-VDJ. A male, five-year- old lion (case no. 24, sample no. 27), that died from a metastasizing osteoblastic osteosarcoma, also showed signs of inflammation in the stomach and the intestines, accompanied by amyloid deposits in intestinal lymph nodes. The clonality assay resulted in a B-cell clonality.

Previous validation studies have shown diagnostic sensitivity of 70 % and specificity of 90 %, an overall diagnostic accuracy of 77 %, and positive and, negative predictive value of 93 % and 60 %, respectively (Hammer et al., 2017).

There are multiple reasons why this method of testing may return false positive results. According to Clonality Guidelines, established by Keller and co-authors in 2016, so-called benign clonal expansion can be detected under certain circumstances as clonal expansions in response to antigenic stimulation, canonical rearrangements and unspecific amplification. Clonal expansion in response to antigenic stimulation is admittedly rare, but a possible disproportion of the proliferation process showing a limited antigen receptor repertoire can lead to a monoclonal peak (Keller et al., 2016). Neoplastic growth, as the possible cause here in three cases implicates immune response, and therefore proliferation and migration of lymphocytes provide a possible explanation for a monoclonal clonality assay outcome.

A lynx (case no. 10, sample no. 13) and a wildcat (case no. 7, sample no. 10), both had a positive result for B-cell clonality. Unfortunately, histopathological examination was limited due to the poor tissue condition after freezing. Although limited, the authors are confident to say that there were no lesions pointing towards a round cell tumor. The wildcat suffered from feline infectious peritonitis and showed pathognomonic lesions for this entity, which could be seen, even if poorly, in histopathology. This increase in inflammatory cells might have altered the results.

In all tested cases and samples, there were 37 samples that showed a histological diagnosis not pointing towards neoplasia and a polyclonal result in clonality testing representing true negative clonality data (Fig. 1, Tab. 2, Suppl. Tab. S3). Thus, we conclude that, the diagnostic sensitivity and specificity of the clonality assay for this cohort of non-domestic felines were 100 % and 88 %, respectively.

Finally, the overall diagnostic accuracy was 89 %. Compared to previously published data derived from a cohort of domestic cats, the application of this clonality testing set-up resulted in increased sensitivity and accuracy because of the absence of false negative clonality results in the studied cases (Hammer et al., 2017).

4 Conclusion

In conclusion, we could verify that the domestic feline-specific primer sets, targeting complete IGH-VDJ and TRG-VJ antigen gene rearrangements, can successfully be applied in non-domestic feline species. We were able to detect 100 % of the histopathological confirmed lymphoma cases, including two T-cell lymphomas and one combined B- and T-cell lymphoma case. Thirty-seven out of 45 negative histopathology samples were confirmed negative, whereas five of these animals happened to have a clonal outcome. The outcome of this study resulted in a diagnostic sensitivity of 100 %, a diagnostic specificity of 88 %, and a diagnostic accuracy of 89 %. However, efforts are still made to increase the accuracy of feline IGH and TRG primers. Recently, Rout et al. (2019) developed novel primers targeting complete IGH-VDJ and TRG rearrangements, as well as incomplete IGH-DJ, kappa deleting element (Kde), and immunoglobulin lambda light chain (IGL) gene rearrangements (Rout et al., 2019). In our lab, we are currently testing these primers comparatively, to evaluate their sensitivity and specificity (Welter et al., unpublished data).

Although clonality testing is a very powerful auxiliary tool in lymphoma diagnostics, it is still mandatory to interpret clonality patterns together with the patient's history, clinical findings and the histopathological examination. The large number of tested samples in this cohort together with the results obtained in this study could help to establish the PCR-based clonality assay as a future-oriented approach for feline lymphoma diagnostics in zoos and animal parks as well as in future evaluations for felid wildlife.

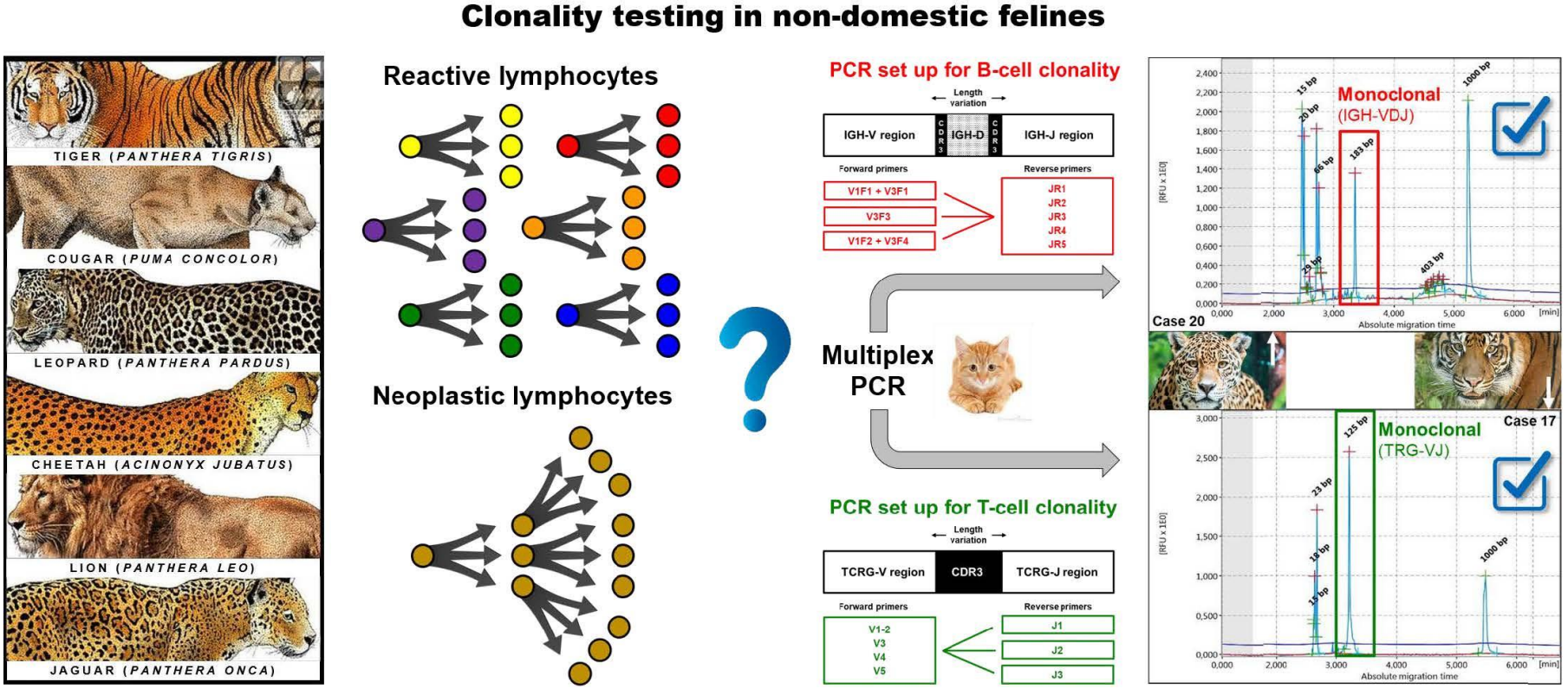


Fig. 3. Graphical abstract

5 Zusammenfassung

Das feline Lymphom, der häufigste maligne Tumor bei Hauskatzen, wird zunehmend auch bei nicht-domestizierten Katzen diagnostiziert. Vor allem bei afrikanischen Löwen (Panthera leo) wurden größere Kohorten untersucht. Der Goldstandard für die Diagnose eines Lymphoms ist die histopathologische Beurteilung. Eine weitere Diagnostikmethode ist die PCR for Antigen Receptor Gene Rearrangement (PARR). Um die Diagnose auf molekularer Ebene zu unterstützen, ist der PCR-basierte Klonalitätstest darauf ausgelegt, zwischen reaktiven und neoplastischen Lymphozytenpopulationen zu unterscheiden. Im Allgemeinen werden PARR-Primer verwendet, um die unterschiedlichen Gen- Anordnungen der schweren Immunglobulin-Kette V-D-J (IGH-VDJ) und der T-Zell-Rezeptor-Gamma-V-J-Kette (TRG-VJ) nachzuweisen. In dieser Studie validierten wir, die in der Routinediagnostik von Hauskatzen verwendeten Primer-Sets, für die Anwendung bei nicht domestizierten Katzen. Klonalitätstests wurden an 41 Individuen bzw. acht unterschiedlichen Katzenarten durchgeführt. Die Ergebnisse wurden zusammen mit der klinischen Vorgeschichte und ihrer Pathologie interpretiert. Insgesamt konnte die Klonalität bei acht nicht-domestizierten Katzen (19,4 %) nachgewiesen werden, darunter drei Tiere mit histopathologisch bestätigten Lymphomen. Diese Ergebnisse bestätigen die erfolgreiche Anwendung von den, für Hauskatzen spezifischen PARR-Primern, bei nicht-domestizierten Katzenarten. Die diagnostische Sensitivität und Spezifität des Klonalitätstests lagen bei 100 % bzw. 88 % die diagnostische Gesamtgenauigkeit bei 89 %. Die große Anzahl getesteter Proben in dieser Kohorte könnte, zusammen mit den in dieser Studie erzielten Ergebnissen, dazu beitragen, den PCR-basierten Klonalitätstest als zukunftsweisenden Ansatz für die Diagnostik von Lymphomen bei Katzen in Zoos und Tierparks, sowie für zukünftige Auswertungen bei wilden Feliden, zu etablieren.

6 Summary

Feline lymphoma, the most common malignant tumor in domestic cats, is also increasingly diagnosed in non- domestic felines, most notably African lions (*Panthera leo*). The gold standard for the diagnosis of lymphoma is histopathological evaluation. As an additional tool, the PCR for antigen receptor gene rearrangement (PARR) has been established. To support the diagnosis on a molecular level, the PCR-based clonality assay is designed to distinguish between reactive and neoplastic lymphocyte populations. In general, PARR primers are used to target complete immunoglobulin heavy chain V-D-J (IGH-VDJ) and T-cell receptor gamma V-J (TRG-VJ) chain gene rearrangements. In this study, we validated the primer sets used in routine diagnostics of domestic cats for the application in non-domestic felines. Clonality testing was used in 41 non-domestic feline species and the results were interpreted in the light of their clinical history and their pathology. In total, clonality could be detected in 8 non-domestic felines (19.4 %), including 3 lymphoma cases confirmed by histopathology. These results confirmed the successful application of domestic feline-specific PARR primers in non-domestic feline species. Diagnostic sensitivity and specificity of the clonality assay were 100 % and 88 %, respectively. Finally, the overall diagnostic accuracy was 89 %.

7 Abbreviations

bp	Base pairs
D	Diversity
DSLL	Diffuse Small Lymphocyte Lymphoma
fAR	Feline androgen receptor
FeLV	Feline Leukemia Virus
Fig.	Figure
FIV	Feline Immunodeficiency Virus
gDNA	Genomic DNA
IGH	Immunoglobulin heavy chain
IGL	Lambda light chain
J	Joining
Kde	Kappa deleting element
No.	Number
PARR	PCR for Antigen Receptor Gene Rearrangement
Suppl.	Supplementary
Tab.	Table
V	Variable

8 References

Gress, V., Wolfesberger, B., Fuchs-Baumgartinger, A., Nedorost, N., Saalmüller, A., Schwendenwein, I., Rütgen, B. C., Hammer S. E., 2016. 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. doi: 10.1016/j.rvsc.2016.07.004.

Hammer, S. E., Groiss, S., Fuchs-Baumgartinger, A., Nedorost, N., Gress, V., Luckschander-Zeller, Saalmüller, A., Schwendenwein, I., Rütgen, B. C., 2017. Characterization of a PCR-based lymphocyte clonality assay as a complementary tool for the diagnosis of feline lymphoma. Vet. Comp. Oncol. 15, 1354-1369. doi: 10.1111/vco.12277.

Harrison, T. M., McKnight, C. A., Sikarskie, J. G, Kitchell, B. E., Garner, M. M., Raymond, J. T., Fitzgerald, S. D., Valli, V. E., Agnew, D., Kiupel, M., 2010. Malignant lymphoma in african lions (panthera leo). Vet. Pathol. 47 (5), 952-957. doi: 10.1177/0300985810375054.

Jung, D., Giallourakis, C., Mostoslavsky, R., Alt, F. W., 2006. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. Annu. Rev. Immunol. 24, 541-570. doi: 10.1146/annurev.immunol.23.021704.115830.

Keller, S. M., Vernau, W., Moore, P. F., 2016. Clonality Testing in Veterinary Medicine: A Review With Diagnostic Guidelines. Vet. Pathol. 53 (4), 711-725. doi: 10.1177/0300985815626576.

Keresztes, M. A., Henrich, M., Baloi, P., Gerst, S., Rudnick, J., Langenstein, J., Moritz, A., Bauer, N., 2017. Nonregenerative immune-mediated anemia associated with a diffuse large B-cell lymphoma in a captive jaguar (Panthera onca). Vet. Clin. Pathol. 46, 597-604. doi: 10.1111/vcp.12542.

Langerak, A. W., Groenen, P. J. T. A., Brüggemann, M., Beldjord, K., Bellan, C., Bonello, L. , Boone, E., Carter, G.I., Catherwood, M., Davi, F., Delfau- Larue, M., Diss, T., Evans, P. A. S., Gameiro, P., Garcia Sanz, R., Gonzalez, D., Grand, D., Hakannson, A., Hummel, M., Liu, H., Lombardia, L., Macintyre, E.A., Milner, B. J., Montes- Moreno, S., Schuuring, E., Spaargaren, M., Hodges, E., van Dongen, J. J. M., 2012. EuroClonality/BIOMED-2 guidelines for

interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. Leukemia 26 (10), 2159-2171. doi: 10.1038/leu.2012.246.

Marker, L., Munson, L., Basson, P. A., Quaokenbush, S., 2003. Multicentric T-cell lymphoma associated with feline leukemia virus infection in a captive Namibian cheetah (Acinonyx jubatus). J. Wildl. Dis. 39(3), 690-695.

Mochizuki, H., Nakamura, K., Sato, H., Goto-Koshino, Y., Sato, M., Takahashi, M., Fujino, Y., Ohno, K., Uchida, K., Nakayama, H., Tsujimoto, H., 2011. Multiplex PCR and Genescan analysis to detect immunoglobulin heavy chain gene rearrangement in feline B-cell neoplasms. Vet. Immunol. Immunopathol. 143, 38-45. doi: 10.1016/j.vetimm.2011.05.030.

Mochizuki, H., Nakamura, K., Sato, H., Goto-Koshino, Y., Sato, M., Takahashi, M., Fukushima, K., Nakashima K., Fujino, Y., Ohno, K., Nakayama, H., Tsujimoto H., 2012. GeneScan analysis to detect clonality of T-cell receptor γ gene rearrangement in feline lymphoid neoplasms. Vet. Immunol. Immunopathol. 145, 402-409. doi: 10.1016/j.vetimm.2011.12.015.

Poli, A., Abramo, F., Cavicchio, P., Bandecchi P., Ghelardi E., Pistello M., 1995. Lentivirus Infection in an African Lion: A Clinical, Pathologic and Virologic Study. J. Wildl. Dis. 31(1), 70-74.

Rout, E. D., Burnett, R. C., Yoshimoto, J. A., Avery, P. R., Avery, A. C., 2019. Assessment of immunoglobulin heavy chain, immunoglobulin light chain, and T-cell receptor clonality testing in the diagnosis of feline lymphoid neoplasia. Vet. Clin. Pathol. 48 Suppl 1:45-58. doi: 10.1111/vcp.12767.

Stockham, S.L., Scott, M.A., 2008. Introductory Concepts, in: Stockham, S.L., Scott, M.A. (Eds.), Fundamentals of Veterinary Clinical Pathology. Blackwell Publishing, Ames, Iowa, USA, pp. 38-39.

van Dongen, J. J. M., Langerak, A. W., Brüggemann, M., Evans, P. A. S., Hummel, M., Lavender, F. L., Delabesse, E., Davi, F., Schuuring, E., Garcia- Sanz, R., van Krieken, J. H. J. M., Droese, J., Gonzalez, D., Bastard, C., White, H. E., Spaargaren, M., Gonzalez, M., Parreira, A., Smith, J. L., Morgan, G.J., Kneba, M., Macintyre, E. A., 2003. 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 (12), 2257-2317. doi: 10.1038/sj.leu.2403202.

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10 Appendix

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Suppl. Tab. S2: DNA quality assessment of 41 non-domestic feline individuals (equal to 45 gDNA samples) analyzed in this study.

Suppl. Tab. S3: Results of the GeneScan analysis of complete IG heavy chain (IGH) VDJ and TCR gamma (TRG) VJ gene rearrangements in 41 non-domestic feline individuals (equal to 45 gDNA samples) analyzed in this study.

Suppl. Tab. S1: Full case history of 41 non-domestic feline individuals (equal to 45 gDNA samples) analyzed in this study.

Case number	Sample number	Sampling date	Common Name	Taxonomic Name	Gender	Age	Origin	Localization	Lymphoma[a]	Cause of death
1	1	2010	European wildcat	*Felis silvestris*	m	1 year	zoo collection	lymph node	No	suffocation
2	2	2015	European wildcat	*Felis silvestris*	f	6 years	zoo collection	lymph node	No	culling
3	3	2016	European wildcat	*Felis silvestris*	m	N/d	wild	spleen	No	FIP
4	4	2016	European wildcat	*Felis silvestris*	f	10 years	zoo collection	spleen	No	renal failure
5	5	2020	European wildcat	*Felis silvestris*	f	juvenile	zoo collection	lymph node	No	euthanasia
	6							spleen		
6	7	2020	European wildcat	*Felis silvestris*	m	adult	zoo collection	lymph node	No	FIP
	8							spleen		
7	9	2020	European wildcat	*Felis silvestris*	f	juvenile	zoo collection	lymph node	No	FIP
	10							spleen		
8	11	N/d	Eurasian lynx	*Lynx lynx*	N/d	N/d	N/d	lymph node	No	N/d
9	12	2012	Eurasian lynx	*Lynx lynx*	f	juvenile	wild	lymph node	No	trauma, hit by car
10	13	2013	Eurasian lynx	*Lynx lynx*	f	N/d	wild	lymph node	No	cachexia
11	14	2014	Eurasian lynx	*Lynx lynx*	m	< 1 year	wild	lymph node	No	trauma
12	15	2015	Eurasian lynx	*Lynx lynx*	f	2.5 months	wild	lymph node	No	trauma
13	16	2016	Eurasian lynx	*Lynx lynx*	m	20.5 years	zoo collection	lymph node	No	multiple organ failure
14	17	2016	Eurasian lynx	*Lynx lynx*	m	4 years	zoo collection	spleen	No	not clear
15	18	2016	Eurasian lynx	*Lynx lynx*	f	2 years	wild (national park)	lymph node	No	not clear
16	19	2010	Tiger	*Panthera tigris*	m	15 years	zoo collection	lymph node	Yes	euthanasia
17	20	2017	Tiger	*Panthera tigris*	f	9 years	zoo collection	lymph node	Yes	lymphoma
18	21	2019	Tiger	*Panthera tigris*	f	juvenile	zoo collection	spleen	No	septicemia
19	22	2019	Tiger	*Panthera tigris*	f	juvenile	zoo collection	spleen	No	euthanasia
20	23	2010	Jaguar	*Panthera onca*	m	17 years	zoo collection	lymph node	No	multiple organ failure
21	24	2015	Jaguar	*Panthera onca*	f	22 years	zoo collection	lymph node	No	euthanasia
22	25	2015	Jaguar	*Panthera onca*	f	22 years	zoo collection	lymph node	No	euthanasia

Case number	Sample number	Sampling date	Common Name	Taxonomic Name	Gender	Age	Origin	Localization	Lymphoma[a]	Cause of death
23	26	2017	Lion	*Panthera leo*	m	18 years	zoo collection	lymph node	No	euthanasia
24	27	2018	Lion	*Panthera leo*	m	5 years	zoo collection	lymph node	No	euthanasia
25	28	2019	Lion	*Panthera leo*	f	neonate	zoo collection	spleen	No	dystocia
	29							lymph node		
26	30	2015	Leopard	*Panthera pardus*	m	21.5 years	zoo collection	lymph node	No	euthanasia
27	31	2010	Cougar	*Puma concolor*	m	1 day	zoo collection	lymph node	No	still birth
28	32	2010	Cougar	*Puma concolor*	f	1 day	zoo collection	lymph node	No	dystocia
29	33	2017	Cougar	*Puma concolor*	f	7 years	zoo collection	lymph node	No	septicemia
30	34	2010	Cheetah	*Acinonyx jubatus*	m	9 years	zoo collection	lymph node	No	drowning
31	35	2010	Cheetah	*Acinonyx jubatus*	f	15 years	zoo collection	lymph node	No	circulatory collapse
32	36	2010	Cheetah	*Acinonyx jubatus*	f	3 days	zoo collection	spleen	No	septicemia
33	37	2011	Cheetah	*Acinonyx jubatus*	f	10 years	zoo collection	spleen	Yes	multiple organ failure
34	38	2014	Cheetah	*Acinonyx jubatus*	f	neonate	zoo collection	spleen	No	still birth
35	39	2015	Cheetah	*Acinonyx jubatus*	f	13 years	zoo collection	spleen	No	euthanasia
36	40	2015	Cheetah	*Acinonyx jubatus*	f	6 months	zoo collection	lymph node	No	drowning
37	41	2015	Cheetah	*Acinonyx jubatus*	f	8 years	zoo collection	lymph node	No	euthanasia
38	42	2016	Cheetah	*Acinonyx jubatus*	m	2 years	zoo collection	lymph node	No	euthanasia
39	43	2017	Cheetah	*Acinonyx jubatus*	m	14 years	zoo collection	lymph node	No	malabsorption syndrome
40	44	2017	Cheetah	*Acinonyx jubatus*	N/d	N/d	wild	spleen	No	culling
41	45	2019	Cheetah	*Acinonyx jubatus*	m	9 years	zoo collection	spleen	No	euthanasia

f, female; m, male; FIP, Feline Infectious Peritonitis; N/d, not determined.

[a]based on the histopathological examination at the Research Institute of Wildlife Ecology (Vetmeduni Vienna).

True positive cases.

True negative cases.

Case history
asphyxia - suffocation from chicken bone
enteritis, encephalomyelitis, heart failure with liver congestion, hyperplasia of the adrenals
FIP
chronic renal failure, uraemia
fibrinous- purulent bronchopneumonia, FCoV- infection, morphological signs of FIP
FIP, purulent bronchitis
FIP, cat flu possible
N/d
cachexia, trauma (hit by car) with multiple fractures and ruptures of spleen and stomach
cachexia, starved to death, focal pneumonia
trauma (hematoma in muscles and brain, lesion of the first cervical vertebra)
massive trauma, multiple rip fractures, lung hemorrhage, liver fissure
dermal apocrine gland adenocarcinoma, fibrosis of the myocardium, renal sclerosis
multiple skin lesions, old fractures
potential heart failure, chronic nephritis
leucocytosis in intestines and parenchymatous organs, lymphoma, chronic interstitial and gomerulonephritis, spondylosis
thymus lymphoma with metastases in the intestines, mild hepatitis, beginning degenerative heart vessel changes
sanguineous meningoencephalitis, enteritis, tubulonephrosis, anemia
sepsis, sanguienous meningoencephalitis, hepatitis, splenitis, nephritis, myocarditis, enteritis
bronchpneumonia, chronic heart and renal failure
mastocytoma with metastases, eosinophilic syndrome, severe arthrosis, nephrosclerosis
mamma carcinoma, dilatation of the right heart, kidney sclerosis, beginning amyloidosis

Case history
chronic heart failure, hepatic- cystadenoma, obstipation of the colon, abscess and wounds at the right hind limb
metastasizing osteoblastic osteosarcoma
dystocia, aspiration of amniotic liquor, bleeding into the abdominal cavity
multifocal suppurative pneumonia, renal sclerosis, cholangioma, gastritis, pancreatic islet cell tumor, nodular adrenocortical hyperplasia, follicular carcinoma of the thyroid gland
still birth
dystocia, aspiration of amniotic liquor
necrosis of the intestinal wall, glomerulonephritis, haemorrhage in different organs, haemorrhagic pneumonia
chronic heart failure, chronic lung congestion and edema, chronic renal failure, rhinitis, hyperemia
obstruction of the esophagus with piece of meat, uraemia, chronic obstipation of the colon
septicemia (lack of colostrum)
chronic nephritis, nephrosis, chronic ulcerative gastritis, enterocolitis, lymphoma, focal bleedings in the brain
still birth, alveolar histiocytosis
multifocal mineralization of the lung, stomach, pancreas and kidneys, beginning amyloidosis of liver and kidney, sclerosis of stomach and large intestine, chronic renal degeneration, moderate lymphoplasmacytic enterocolitis, tubulo- acinar adenoma of the pancreas (metastatic calcification secondary to the chronic renal problems)
death from drowning, enteritis, liver capsule fibrosis, proteinuria
chronic nephritis, gastritis, hypertrophy of the left heart, anemia
non- purulent encephalitis, myocarditis, hepatitis, endoparasitosis
malabsorption syndrome, cachexia, chronic heart failure, chronic degenerative arthritis
culled
feline coronavirus positive, history of epileptic seizuring, adiposity, chronic heart failure, endoparasitosis, chronic gastritis, gastric ulcer,

Suppl. Tab. S2: DNA quality assessment of 41 non-domestic feline individuals (equal to 45 gDNA samples) analyzed in this study.

Case number	Sample number	Genomic DNA concentration (ng/µl)	quality 260/280	quality 260/230	fAR
1	1	514.95	1.89	2.26	+
2	2	642.00	1.87	2.26	+
3	3	135.65	1.92	2.33	+
4	4	336.20	1.90	2.22	+
5	5	512.10	1.89	2.32	+
	6	75.55	2.02	2.37	+
6	7	637.40	1.87	2.26	+
	8	88.60	2.00	2.19	+
7	9	285.60	1.91	2.34	+
	10	44.65	2.16	2.23	+
8	11	412.50	1.89	2.33	+
9	12	480.65	1.88	2.29	+
10	13	67.15	1.96	2.18	+
11	14	190.60	1.88	2.28	+
12	15	513.90	1.87	2.27	+
13	16	427.30	1.88	2.35	+
14	17	105.95	1.99	2.31	+
15	18	1166.10	1.89	2.29	+
16	19	213.70	1.90	2.28	+
17	20	526.15	1.85	2.15	+
18	21	783.25	1.91	2.34	+
19	22	602.35	1.87	2.32	+
20	23	444.15	1.93	2.24	+

Case number	Sample number	Genomic DNA concentration (ng/µl)	quality 260/280	quality 260/230	fAR
21	24	150.10	1.93	2.06	+
22	25	719.85	1.89	2.30	+
23	26	489.65	1.87	2.25	+
24	27	594.35	1.87	2.28	+
25	28	531.70	1.88	2.32	+
	29	545.05	1.88	2.32	+
26	30	1083.30	1.85	2.03	+
27	31	93.35	1.91	2.20	+
28	32	206.25	1.90	2.37	+
29	33	496.50	1.89	2.31	+
30	34	147.10	1.91	2.01	-
31	35	192.60	1.88	2.27	-
32	36	98.10	1.92	2.04	-
33	37	298.05	1.90	2.28	-
34	38	281.80	1.86	2.22	-
35	39	34.30	2.01	2.63	+
36	40	234.65	1.81	1.89	+
37	41	787.75	1.90	2.32	-
38	42	220.50	1.91	2.09	-
39	43	375.60	1.89	2.35	+
40	44	45.60	1.98	2.17	+
41	45	314.65	1.90	2.31	+

fAR, feline androgen receptor.

Suppl. Tab. S3: Results of the GeneScan analysis of complete IG heavy chain (IGH) VDJ and TCR gamma (TRG) VJ gene rearrangements in 41 non-domestic feline individuals (equal to 45 gDNA samples) analyzed in this study

		Primer sets											
Case	Sample	IGH-VDJ							TRG-VJ				
number	number	V1F2[a]	V3F3[a]	V3F4[a]	V1F1[a]	V3F1[a]	V3F2[a]	clonality	TRG-J1[b]	TRG-J2[b]	TRG-J3[b]	clonality	Clonality result
1	1	PC	PC	PC	PC	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
2	2	PC	PC	PC	PC	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
3	3	PSC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
4	4	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
5	5	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
	6	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
6	7	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
	8	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
7	9	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
	10	**MC**	PC	PC	N/a	N/a	N/a	**pos**	PC	PC	N	N/d	**B-cell clonality**
8	11	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
9	12	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
10	**13**	**MC**	PSC	PC	PC	N/a	N/a	**pos**	PC	PC	N	N/d	**B-cell clonality**
11	14	PSC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
12	15	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
13	16	PSC	N	N	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
14	17	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
15	18	PSC	PC	PSC	pc	pc	pc	N/d	PC	PC	N/a	N/d	no clonality
16	**19**	PSC	N	**MC in PC Bg**	N/a	N/a	N/a	**pos**	**MC**	PC	N	**pos**	**B- and T-cell clonality**
17	**20**	N	PSC	N	N/a	N/a	N/a	N/d	PC	**MC in PC Bg**	N	**pos**	**T-cell clonality**
18	21	PSC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
19	22	PSC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
20	**23**	**MC**	N	N	N/a	N/a	N/a	**pos**	PC	PC	N	N/d	**B-cell clonality**

		Primer sets											
Case	Sample	IGH-VDJ							TRG-VJ				
number	number	V1F2[a]	V3F3[a]	V3F4[a]	V1F1[a]	V3F1[a]	V3F2[a]	clonality	TRG-J1[b]	TRG-J2[b]	TRG-J3[b]	clonality	Clonality result
21	**24**	PSC	**MC**	PC	N/a	N/a	N/a	**pos**	PC	**MC**	N	**pos**	**B- and T-cell clonality**
22	25	PSC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
23	26	PSC	PC	PC	N/a	PC	PC	N/d	PC	PC	N/a	N/d	no clonality
24	**27**	PC	PC	PC	**MC in PC Bg**	PC	PC	**pos**	PC	PC	N	N/d	**B-cell clonality**
25	28	PSC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
	29	PSC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
26	30	PSC	N	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
27	31	PSC	pc	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
28	32	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
29	33	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
30	34	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
31	35	PC	N	N	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
32	36	PC	PC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
33	**37**	PSC	PC	PC	N/a	N/a	N/a	N/d	PC	**MC**	N	**pos**	**T-cell clonality**
34	38	PSC	N	N	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
35	39	PSC	PSC	PC	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
36	40	N	N	N	N/a	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
37	41	PC	PC	PC	PC	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
38	42	PC	PC	PC	PC	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
39	43	PSC	PC	PC	PC	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
40	44	PC	PC	PC	PC	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality
41	45	PC	PC	PC	PC	N/a	N/a	N/d	PC	PC	N/a	N/d	no clonality

MC, monoclonal; PC, polyclonal; PSC, pseudoclonal; Bg, background; N/a, not analyzed; N/d, clonality not detected;

N, negative PCR reaction. [a]Mochizuki et al. (2011); [b]Mochizuki et al. (2012)

Confirmed lymphoma based on the histopathological examination at the Research Institute of Wildlife Ecology (Vetmeduni Vienna).

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First-time application of a PCR-based clonality assay in a large cohort of non-domestic felines

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ABSTRACT

Feline lymphoma, one of the most important malignant tumors in domestic cats, is also increasingly diagnosed in non-domestic felines, most notably, African lions (*Panthera leo*). The gold standard for the diagnosis of lymphoma is histopathological evaluation. As an additional tool, the PCR for antigen receptor gene rearrangement (PARR) has been established. To support the diagnosis on a molecular level, the PCR-based clonality assay is designed to distinguish between reactive and neoplastic lymphocyte populations. In general, PARR primers are used to target complete immunoglobulin heavy chain V-D-J (IGH-VDJ) and T-cell receptor gamma V-J (TRG-VJ) chain gene rearrangements. In this study, we validated the primer sets used in routine diagnostics of domestic cats for the application in non-domestic felines. Clonality testing was used in 41 non-domestic feline species and the results were interpreted in the light of their clinical history and their pathology. In total, clonality could be detected in 8 non-domestic felines (19.4%), including 3 lymphoma cases confirmed by histopathology. These results confirmed the successful application of domestic feline-specific PARR primers in non-domestic feline species. Diagnostic sensitivity and specificity of the clonality assay were 100% and 88%, respectively. Finally, the overall diagnostic accuracy was 89%.

1. Introduction

Lymphoma, the most common tumor in domestic cats, is increasingly diagnosed in non-domestic felines. Although the exact prevalence of lymphoma in different feline species in unknown, the occurrence of malignant lymphoma in captive African lions (*Panthera leo*), is well-reported (Harrison et al., 2010). In contrast to domestic cats, the African lion is more likely to suffer from T- cell lymphoma. The main clinical signs include weight loss and splenomegaly and, typical for T-cell lymphoma, spleen, liver and regional lymph node involvement. Although there is a confirmed correlation between lymphoma and Feline Leukemia Virus (FeLV) as well as Feline Immunodeficiency Virus (FIV) in domestic cats, there is no such association in lions.

Apart from lions, lymphoma cases have been reported in other feral feline species including cheetahs (*Acinonyx jubatus*) and jaguars (*Panthera onca*) (Marker et al., 2003; Keresztes et al., 2017).

Keresztes et al. (2017) reported a non-regenerative immune-mediated anemia associated with a diffuse large B-cell lymphoma in a captive jaguar (*Panthera onca*) (Keresztes et al., 2017). The animal presented with anorexia and hemostasis dysfunction prior to the diagnosis. The primary sites of neoplastic growth were the spleen, the liver and, in contrary to the reported cases of T-cell lymphomas, peripheral lymph nodes.

As in African lions, a multicentric T-cell lymphoma has been reported in a captive Namibian cheetah (*Acinonyx jubatus*). In this case, which was associated with Feline Leukemia Virus infection, the liver, spleen, lymph nodes and other organs proved to be infiltrated with neoplastic T-lymphocytes (Marker et al., 2003).

In 1991, a captive lion tested positive for feline immunodeficiency virus (FIV), after showing typical clinical signs for a lentivirus infection (Poli et al., 1995). In addition to other abnormalities, lymphoma was detected and classified as Diffuse Small Lymphocytic Lymphoma (DSLL).

Basic diagnostics in the above cases included common methods such as physical examination, total blood count, blood chemistry and further

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diagnostics like ultrasound, fine needle aspiration, biopsies and radiography. The gold standard for the diagnosis of feline lymphoma is the histopathological and cytological examination of biopsy samples.

As a useful adjunct diagnostic tool, the PCR-based lymphocyte clonality assay has been tested, improved and established (van Dongen et al., 2003). The method is based on the different characteristics of reactive and malignant lymphocytes. Neoplastic B- and T-cells, originating from one single transformed cell, can be distinguished from benign lymphocytes by their antigen receptor gene rearrangements (Keller et al., 2016). The different assemblies of the immunoglobulin heavy chain (IGH) variable region and the T-cell receptor gamma chain (TRG) originate by random recombination of variable (V), diversity (D), and joining (J) regions (Jung et al., 2006). This entails that all tumorous cells contain a unique clonal IGH or TRG gene rearrangement (Keller et al., 2016). To interpret the clonality of lymphocyte populations, the PCR-products are assessed using multicapillary gel electrophoresis, a Gene Scanning device. The clonality patterns illustrate the difference between reactive and neoplastic lymphocytes and could therefore serve as an adjunct tool in the diagnosis of lymphoma in non-domestic felines.

Therefore, the aim of this study was to test the applicability of the primer sets used to assess clonality in domestic cats, for wild, namely non-domestic felines. In this study, clonality could be detected in 8 non-domestic felines, including 3 lymphoma cases confirmed by histopathology. These results point towards the specific usability of domestic feline-specific IGH-VDJ or TRG-VJ primers in non-domestic feline species.

2. Material and methods

2.1. Case selection and origin of sample material

Between 2010 and February 2020, spleen and lymph node tissue samples were taken from 41 exotic felids during routine post-mortem examinations at the Research Institute of Wildlife Ecology (Vetmeduni Vienna). After necropsy, the specimens were stored in small containers and frozen at −80 °C. Included in this study are eight different non-domestic feline species: European wildcat (*Felis silvestris*), Eurasian lynx (*Lynx lynx*), tiger (*Panthera tigris*), jaguar (*Panthera onca*), African lion (*Panthera leo*), leopard (*Panthera pardus*), cougar (*Puma concolor*) and cheetah (*Acinonyx jubatus*). Thirty-three of these animals lived in captivity and seven originated from the wild (Table 1, Supplementary Table S1).

Table 1
Condensed case history of 41 non- domestic feline individuals (equal to 45 gDNA samples) analyzed in this study.

Case number	Sample number	Common name	Age	Origin	Sampling date	Localization	Lymphoma[a]	Cause of death
1	1	European wildcat	1 year	zoo collection	2010	lymph node	No	suffocation
2	2	European wildcat	6 years	zoo collection	2015	lymph node	No	culling
4	3	European wildcat	N/d	wild	2016	spleen	No	FIP
3	4	European wildcat	10 years	zoo collection	2016	spleen	No	renal failure
5	5	European wildcat	juvenile	zoo collection	2020	lymph node	No	euthanasia
	6					spleen	No	
6	7	European wildcat	adult	zoo collection	2020	lymph node	No	FIP
	8					spleen	No	
7	9	European wildcat	juvenile	zoo collection	2020	lymph node	No	FIP
	10					spleen	No	
8	11	Eurasian lynx	N/d	N/d	N/d	lymph node	No	N/d
9	12	Eurasian lynx	juvenile	wild	2012	lymph node	No	trauma, hit by car
10	13	Eurasian lynx	N/d	wild	2013	lymph node	No	cachexia
11	14	Eurasian lynx	< 1 year	wild	2014	lymph node	No	trauma
12	15	Eurasian lynx	2.5 months	wild	2015	lymph node	No	trauma
13	16	Eurasian lynx	20.5 years	zoo collection	2016	lymph node	No	multiple organ failure
14	17	Eurasian lynx	4 years	zoo collection	2016	spleen	No	Not clear
15	18	Eurasian lynx	2 years	wild (national park)	2016	lymph node	No	Not clear
16	19	Tiger	15 years	zoo collection	2010	lymph node	Yes	euthanasia
17	20	Tiger	9 years	zoo collection	2017	lymph node	Yes	lymphoma
18	21	Tiger	juvenile	zoo collection	2019	spleen	No	septicemia
19	22	Tiger	juvenile	zoo collection	2019	spleen	No	euthanasia
20	23	Jaguar	17 years	zoo collection	2010	lymph node	No	multiple organ failure
21	24	Jaguar	22 years	zoo collection	2015	lymph node	No	euthanasia
22	25	Jaguar	22 years	zoo collection	2015	lymph node	No	euthanasia
23	26	Lion	18 years	zoo collection	2017	lymph node	No	euthanasia
24	27	Lion	5 years	zoo collection	2018	lymph node	No	euthanasia
25	28	Lion	neonate	zoo collection	2019	spleen	No	dystocia
	29					lymph node	No	
26	30	Leopard	21.5 years	zoo collection	2015	lymph node	No	euthanasia
27	31	Cougar	1 day	zoo collection	2010	lymph node	No	still birth
28	32	Cougar	1 day	zoo collection	2010	lymph node	No	dystocia
29	33	Cougar	7 years	zoo collection	2017	lymph node	No	septicemia
30	34	Cheetah	9 years	zoo collection	2010	lymph node	No	drowning
31	35	Cheetah	15 years	zoo collection	2010	lymph node	No	circulatory collapse
32	36	Cheetah	3 days	zoo collection	2010	spleen	No	septicemia
33	37	Cheetah	10 years	zoo collection	2011	spleen	Yes	multiple organ failure
34	38	Cheetah	neonate	zoo collection	2014	spleen	No	still birth
35	39	Cheetah	13 years	zoo collection	2015	spleen	No	euthanasia
36	40	Cheetah	6 months	zoo collection	2015	lymph node	No	drowning
37	41	Cheetah	8 years	zoo collection	2015	lymph node	No	euthanasia
38	42	Cheetah	2 years	zoo collection	2016	lymph node	No	euthanasia
39	43	Cheetah	14 years	zoo collection	2017	lymph node	No	malabsorption syndrome
40	44	Cheetah	N/d	wild	2017	spleen	No	culling
41	45	Cheetah	9 years	zoo collection	2019	spleen	No	euthanasia

N/d, not determined; FIP, Feline infectious peritonitis.
[a] Based on the histopathological examination at the Research Institute of Wildlife Ecology (Vetmeduni Vienna).

2.2. Histopathological examination

As part of the dissection, the organs were routinely sampled and put in 7% neutral buffered formalin for fixation. Slices of 4 μm thickness were cut from the different tissues and placed onto microscope slides. The slides were stained with hematoxylin and eosin (HE stain; Merck KGaA, Darmstadt, Germany) and examined under the microscope.

2.3. DNA extraction and gDNA quality control

Total genomic DNA (gDNA) was extracted by using the E.Z.N.A Tissue DNA Kit (Omega Biotech, Norcross, Georgia) following the manufacturer's instructions. Between 30 and 60 mg of tissue were minced and further processed as follows. The minced tissue was transferred to a 1.5 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany) and 400 μL of TL buffer were added. After the addition of 25 μL of OB Protease Solution, the mixture was thoroughly vortexed. The tissue-buffer solution was incubated overnight at 55 °C in a shaking water bath, to enable complete tissue lysis. For RNA digestion, 4 μL RNase A (100 mg/mL) was added and the samples were incubated for 2 min at room temperature. For subsequent cell lysis, 420 μL BL buffer were added, followed by another incubation step at 70 °C for 10 min. Next, the adjusted amount of 420 μL of 100% ethanol was added and the mixture thoroughly vortexed. The whole sample was transferred to an assembled HiBind®DNA Mini Column and centrifuged at 13,200 rpm (Microcentrifuge, Eppendorf AG) for 1 min. After discarding the flow-through, 500 μL of HBC Buffer were added followed by a centrifuge step at 13,200 rpm for 1 min. Again, the flow-through was discarded and two washing steps were performed by adding 700 μL DNA Wash Buffer followed by a centrifuge step at 13,200 rpm for 1 min. To dry the mini column, the empty HiBind®DNA Mini Column assembly was centrifuged at 13,200 rpm for 2 min. The mini column was transferred to another 1.5 mL Eppendorf tube (Eppendorf AG). Finally, gDNA was eluted from the mini column by adding 100–200 μL pre-heated (70 °C) Elution Buffer and the entire sample was left at room temperature for 2 min and centrifuged at 10,000 rpm for 2 min. This step was repeated by reloading the flow-through to the mini column to perform a second elution step.

The concentration as well as the quality of the extracted gDNA were assessed with the Nano Drop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) in pedestal mode. Per gDNA sample, at least two measurements were performed, making sure that the two values would not differ more than 5 ng/μL. The threshold was set to 30 ng/μL with desired 260/280 ratios of 1.8–2.0 and a 260/230 ratios of above or equal two (2.0–2.2).

To evaluate the suitability of the gDNA for the clonality assay, a 189 bp fragment of the feline androgen receptor gene (fAR) was PCR-amplified for each sample using the following primers: forward primer 5'-CACAATGCCGCTACGGGGACCT-3' (Mochizuki et al., 2012) and reverse primer 5'-AGGGGGTCACAGACCCTGACTCG-3' (Mochizuki, pers. com.). The PCR reaction contained 4× AllTaq PCR Master Mix (Qiagen GmbH, Hilden, Germany), 1× CoralLoad® (Qiagen), 10–40 pM of each fAR primer (Eurofins Genomics, Ebersberg, Germany) and 150 ng of eluted gDNA (>30 ng/μL; Supplementary Table S2) as template brought up to 12.5 μL with molecular biology grade water (Qiagen). Additionally, the feline lymphoma cell lines MS4 and FT-1 (Mochizuki et al., 2012; Mochizuki et al., 2011) were used as positive controls and the size of the PCR product was evaluated using standard agarose gel electrophoresis.

2.4. PCR protocol for the Clonality testing

For the amplification of the IGH-VDJ and TRG-VJ gene rearrangements, specific primer sets designed by Mochizuki and co-workers and synthesized by Eurofins Genomics were used (Mochizuki et al., 2012; Mochizuki et al., 2011). In total, six primer sets were applied to target complete IGH-VDJ rearrangements, and a multiplex primer set was used to target TRG specific genes (primer set B in Hammer et al., 2017). IGH-VDJ and TRG-VJ gene rearrangements were amplified as previously described for primer set B (Hammer et al., 2017). After PCR, 10 μL of DNA Dilution Buffer (Qiagen) were added to each PCR reaction and size separated using the QIAxcel Advanced System capillary electrophoresis analyzer with the QIAxcel DNA High Resolution Kit and the QX Alignment Marker 15 bp/1000 bp (Qiagen). The presence and size of obtained PCR products was accurately determined using QIAxcel ScreenGel Software (Qiagen) (Hammer et al., 2017; Gress et al., 2016).

2.5. Interpretation of clonality patterns

The clonality patterns were interpreted using guidelines for clonality testing in veterinary medicine (Keller et al., 2016) and the EuroClonality/BIOMED-2 guidelines for clonality testing in human medicine (Langerak et al., 2012). Identical PCR triplicates verified the reproducibility of the clonality patterns for every sample and PCR reactions not following this rule were defined as pseudoclonal. The patterns were categorized according to their peak morphology. Monoclonal peaks were defined as being at least double the height of the background, whereas polyclonality was characterized by multiple peaks being organized in a bell-shaped curve. Definite single peaks surrounded by a polyclonal setting were defined as monoclonal with a polyclonal background (Hammer et al., 2017; Gress et al., 2016).

Diagnostic sensitivity, diagnostic specificity and accuracy of the test for this non-domestic feline cohort was calculated based on the histopathological diagnosis as a gold standard defining true positive, true negative, false negative and false positive clonality results (Stockham and Scott, 2008).

3. Results and discussion

3.1. Impact of gDNA quality on the results of the PCR-based clonality assay

The 45 gDNA samples tested originated from seven wildcats, eight lynx, four tigers, three jaguars, three lions, one leopard, three cougars and twelve cheetahs with ages ranging from neonatal to twenty-two years of age. For nine animals, the exact ages where unknown. Thirty-three animals belonged to different zoo collections and seven animals originated from the wild. Detailed information about the studied animals including full case history and clinical signs are given in Table 1 and Supplementary Table S1. For three European wildcats (case no. 5, 6 and 7) and one lion (case no. 25), the gDNAs derived from two different tissues, lymph node and spleen.

Genomic DNA concentrations ranged from 34.3 to 1166.1 ng/μL with a mean value of 381.05 ng/μL (Supplementary Table S2). The 260/280 ratio presented with a maximum of 2.16, a minimum of 1.81 and a mean value of 1.91. The results of the 260/230 ratio showed a maximum of 2.63, a minimum of 1.89, next to a mean value of 2.25 (Supplementary Table S2). In total, 45 tissue samples obtained from 41 animals were subjected to the amplification of the 189 bp fragment of the feline androgen receptor gene (fAR), serving as internal PCR control.

Despite 38 positive samples, seven out of the twelve cheetah gDNAs failed in the fAR PCR because of sequence ambiguities at the fAR forward primer-binding site in the cheetah genomes. The gDNA concentrations of the fAR-negative cheetah samples ranged from 98.10 to 787.75 ng/μL, the 260/30 ratios lay within 1.86 and 1.92, and the 260/30 ratios ranged between 2.01 and 2.32, hence complying to the recommended gDNA quality standards as postulated by Hammer and co-authors in 2017 (Hammer et al., 2017). Despite the negative fAR PCR results, we decided to include all twelve cheetahs in this study to ensure we did not to lose valuable scientific information about the cheetah cases. Hence, all 45 non-domestic feline samples (equal to 41 non-domestic feline individuals) were assayed for their IGH-VDJ and TRG-

V-J gene rearrangements by clonality testing (Supplementary Table S2).

3.2. Consistency of clonality patterns with histopathological evidence

After PCR-based lymphocyte clonality assay and subsequent Genescan analysis of the PCR products, eight samples from eight cases [case 7 (localization spleen), 10, 16, 17, 20, 21, 24, 33)] showed clonal results, two of them of T-cell and four of them of B-cell clonality and another two of B- and T-cell clonality (Fig. 1, Table 2, Supplementary Table S3).

Three of these samples (two tigers and one cheetah, case no. 16, 17 and 33, sample no. 19, 20 and 37) are confirmed lymphoma cases, based on histopathology and hence showing a true positive result (Supplementary Table S1). All these animals were kept in zoos and were between nine and fifteen years old. Lymph nodes were used as sample material for two of them (two tigers, case no. 16 and 17, sample no. 19 and 20) and spleen tissue for the third one (cheetah, case no. 33, sample no. 37) (Table 2).

At the dissection, a ten-year-old, female cheetah (case no. 33, sample no. 37) showed, in addition to a lymphoma in the spleen, chronic alterations in the kidneys and stomach, as well as age- related changes of liver, heart, kidneys and brain. The PCR- based clonality assay confirmed a T-cell lymphoma with a distinct monoclonal peak, as shown in Fig. 2.

Furthermore, the method could verify a T-cell lymphoma of a female nine-year-old tiger (case no. 17, sample no. 20) who presented with a thymus lymphoma associated with metastases in the intestines (Fig. 2). The third case, a male fifteen-year-old tiger (case no. 16, sample no. 19) was euthanized after a history of inappetence, hind limb weakness, polydipsia and kidney failure. The following pathological dissection revealed a multicentric lymphatic leukosis in the bowel, and degenerative changes in multiple organs. The clonality assay resulted in a B- and T-cell clonality, supporting the histological findings from the dissection (Fig. 2). The lymphocytes derived from one neoplastic cell result in a monoclonal peak, as they have a homogenous antigen gene rearrangement.

3.3. False positive clonality patterns as compared with the histopathological gold standard

Five samples showed no evidence of neoplastic lymphocytes at histopathology, but monoclonality of B- and/or T-cell antigen receptor gene rearrangements representing false positive clonality results (Fig. 1, Table 2, Supplementary Table S3). A female jaguar, twenty-two years of age (case no. 21, sample no. 24), presented with a mastocytoma with metastases and potential hypereosinophilic syndrome together with other degenerative changes (Supplementary Table S1). Contrary to the

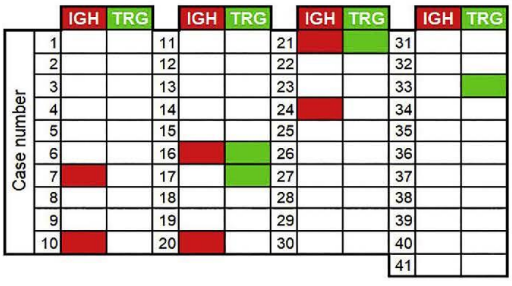

Fig. 1. This heat map summarizes the occurrence of B- and T-cell clonality in 41 non-domestic feline cases as detected with IGH-VDJ and TRG-VJ specific primer sets. Cases 16 and 21 showed B- and T-cell clonality. Only B-cell clonality was found for cases 7, 10, 20 and 24; whereas cases 17 and 33 were positive for T-cell clonality.

Table 2
Summarizing results of 41 non- domestic feline individuals (equal to 45 gDNA samples) analyzed in this study.

Case number	Sample number	Common name	Localization	Clonality	Lymphoma[a]
1	1	European wildcat	lymph node	N/d	No
2	2	European wildcat	lymph node	N/d	No
4	3	European wildcat	spleen	N/d	No
3	4	European wildcat	spleen	N/d	No
5	5	European	lymph node	N/d	No
	6	wildcat	spleen	N/d	No
6	7	European	lymph node	N/d	No
	8	wildcat	spleen	N/d	No
7	9	European	lymph node	N/d	No
	10	wildcat	spleen	B-cell	No
8	11	Eurasian lynx	lymph node	N/d	No
9	12	Eurasian lynx	lymph node	N/d	No
10	13	Eurasian lynx	lymph node	B-cell	No
11	14	Eurasian lynx	lymph node	N/d	No
12	15	Eurasian lynx	lymph node	N/d	No
13	16	Eurasian lynx	lymph node	N/d	No
14	17	Eurasian lynx	spleen	N/d	No
15	18	Eurasian lynx	lymph node	N/d	No
16	19	Tiger	lymph node	B-and T-cell	Yes
17	20	Tiger	lymph node	T-cell	Yes
18	21	Tiger	spleen	N/d	No
19	22	Tiger	spleen	N/d	No
20	23	Jaguar	lymph node	B-cell	No
21	24	Jaguar	lymph node	B- and T-cell	No
22	25	Jaguar	lymph node	N/d	No
23	26	Lion	lymph node	N/d	No
24	27	Lion	lymph node	B-cell	No
25	28	Lion	spleen	N/d	No
	29		lymph node	N/d	No
26	30	Leopard	lymph node	N/d	No
27	31	Cougar	lymph node	N/d	No
28	32	Cougar	lymph node	N/d	No
29	33	Cougar	lymph node	N/d	No
30	34	Cheetah	lymph node	N/d	No
31	35	Cheetah	lymph node	N/d	No
32	36	Cheetah	spleen	N/d	No
33	37	Cheetah	spleen	T-cell	Yes
34	38	Cheetah	spleen	N/d	No
35	39	Cheetah	spleen	N/d	No
36	40	Cheetah	lymph node	N/d	No
37	41	Cheetah	lymph node	N/d	No
38	42	Cheetah	lymph node	N/d	No
39	43	Cheetah	lymph node	N/d	No
40	44	Cheetah	spleen	N/d	No
41	45	Cheetah	spleen	N/d	No

N/d, not detected.
[a] Based on the histopathological examination at the Research Institute of Wildlife Ecology (Vetmeduni Vienna).

findings of the pathology, the outcome of the clonality assay was positive for V3F3 and TRG-J2 primers. The most significant findings of another jaguar, male and 17 years old (case no. 20, sample no. 23), included multiple myelolipomas in the spleen, lymphadenitis and chronic inflammation signs in several organs, whereas the clonality assay revealed a monoclonal peak for the primer targeting IGH-VDJ. A male, five-year- old lion (case no. 24, sample no. 27), that died from a metastasizing osteoblastic osteosarcoma, also showed signs of

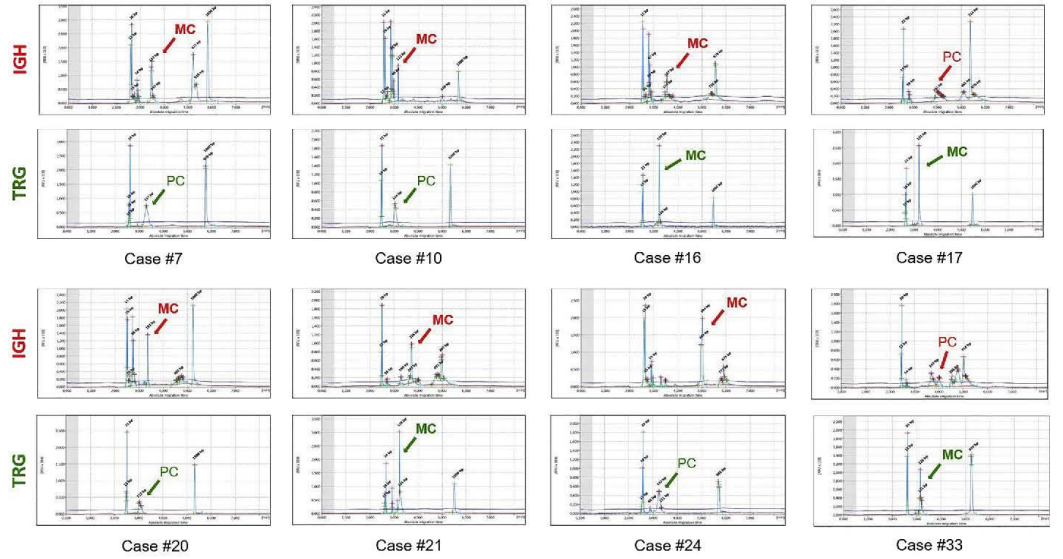

Fig. 2. Representative clonality patterns (electropherograms) of the eight positive non-domestic feline cases 7, 10, 16, 17, 20, 21, 24 and 33 for the IGH-VDJ and TRG-VJ primer sets. Cases 16 and 21 show a monoclonal population in both reaction setups. Cases 7, 10, 20 and 24 have a monoclonal peak in reaction setup IGH and a polyclonal distribution in primer mix TRG. Cases 17 and 33 revealed a monoclonal peak in the TRG-specific reaction mix and a polyclonal distribution with IGH-specific primers. The expected amplicons ranged from ~100 to ~400 bp and primer dimers ranged from 60 to 70 bp. MC, monoclonal; PC, polyclonal; RFU, relative fluorescent units.

inflammation in the stomach and the intestines, accompanied by amyloid deposits in intestinal lymph nodes. The clonality assay resulted in a B-cell clonality.

Previous validation studies have shown diagnostic sensitivity of 70% and specificity of 90%, an overall diagnostic accuracy of 77%, and positive and negative predictive value of 93% and 60%, respectively (Hammer et al., 2017).

There are multiple reasons why this method of testing may return false positive results. According to Clonality Guidelines, established by Keller and co-authors in 2016, so-called benign clonal expansion can be detected under certain circumstances as clonal expansions in response to antigenic stimulation, canonical rearrangements and unspecific amplification. Clonal expansion in response to antigenic stimulation is admittedly rare, but a possible disproportion of the proliferation process showing a limited antigen receptor repertoire can lead to a monoclonal peak (Keller et al., 2016). Neoplastic growth, as the possible cause here in three cases implicates immune response, and therefore proliferation and migration of lymphocytes provide a possible explanation for a monoclonal clonality assay outcome.

A lynx (case no. 10, sample no. 13) and a wildcat (case no. 7, sample no. 10), both had a positive result for B-cell clonality. Unfortunately, histopathological examination was limited due to the poor tissue condition after freezing. Although limited, the authors are confident to say that there were no lesions pointing towards a round cell tumor. The wildcat suffered from feline infectious peritonitis and showed pathognomonic lesions for this entity, which could be seen, even if poorly, in histopathology. This increase in inflammatory cells might have altered the results.

In all tested cases and samples, there were thirty-seven samples that showed a histological diagnosis not pointing towards neoplasia and a polyclonal result in clonality testing representing true negative clonality data (Fig. 1, Table 2, Supplementary Table S3). Thus, we conclude that the diagnostic sensitivity and specificity of the clonality assay for this cohort of non-domestic felines were 100% and 88%, respectively. Finally, the overall diagnostic accuracy was 89%. Compared to previously published data derived from a cohort of domestic cats, the application of this clonality testing set-up resulted in increased sensitivity and accuracy because of the absence of false negative clonality results in the studied cases (Hammer et al., 2017).

4. Conclusion

In conclusion, we could verify that the domestic feline-specific primer sets, targeting complete IGH-VDJ and TRG-VJ antigen gene rearrangements, can successfully be applied in non-domestic feline species. We were able to detect 100% of the histopathological confirmed lymphoma cases, including two T-cell lymphomas and one combined B- and T-cell lymphoma case. Thirty-seven out of 45 negative histopathology samples were confirmed negative, whereas 5 of these animals happened to have a clonal outcome. The outcome of this study resulted in a diagnostic sensitivity of 100%, a diagnostic specificity of 88%, and a diagnostic accuracy of 89%. However, efforts are still made to increase the accuracy of feline IGH and TRG primers. Recently, Rout et al. (2019) developed novel primers targeting complete IGH-VDJ and TRG rearrangements, as well as incomplete IGH-DJ, kappa deleting element (Kde), and immunoglobulin lambda light chain (IGL) gene rearrangements (Rout et al., 2019). In our lab, we are currently testing these primers comparatively, to evaluate their sensitivity and specificity (Welter et al., unpublished data).

Although clonality testing is a very powerful auxiliary tool in lymphoma diagnostics, it is still mandatory to interpret clonality patterns together with the patient's history, clinical findings and histopathological examination. The large number of tested samples in this cohort together with the results obtained in this study could help to establish the PCR-based clonality assay as a future-oriented approach for feline lymphoma diagnostics in zoos and animal parks as well as in future

evaluations for felid wildlife.

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References

van Dongen, J.J.M., Langerak, A.W., Brüggemann, M., Evans, P.A.S., Hummel, M., Lavender, F.L., Delabesse, E., Davi, F., Schuuring, E., García-Sanz, R., van Krieken, J. H.J.M., Droese, J., Gonzalez, D., Bastard, C., White, H.E., Spaargaren, M., Gonzalez, M., Parreira, A., Smith, J.L., Morgan, G.J., Kneba, M., Macintyre, E.A., 2003. 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. https://doi.org/10.1038/sj.leu.2403202.

Gress, V., Wolfesberger, B., Fuchs-Baumgartinger, A., Nedorost, N., Saalmüller, A., Schwendenwein, I., Rütgen, B.C., Hammer, S.E., 2016. 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. https://doi.org/10.1016/j.rvsc.2016.07.004.

Hammer, S.E., Groiss, S., Fuchs-Baumgartinger, A., Nedorost, N., Gress, V., Luckschander-Zeller, Saalmüller, A, Schwendenwein, I., Rütgen, B.C., 2017. Characterization of a PCR-based lymphocyte clonality assay as a complementary tool for the diagnosis of feline lymphoma. Vet. Comp. Oncol. 15, 1354–1369. https://doi.org/10.1111/vco.12277.

Harrison, T.M., McKnight, C.A., Sikarskie, J.G., Kitchell, B.E., Garner, M.M., Raymond, J. T., Fitzgerald, S.D., Valli, V.E., Agnew, D., Kiupel, M., 2010. Malignant lymphoma in african lions (panthera leo). Vet. Pathol. 47, 952–957. https://doi.org/10.1177/0300985810375054.

Jung, D., Giallourakis, C., Mostoslavsky, R., Alt, F.W., 2006. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. Annu. Rev. Immunol. 24, 541–570. https://doi.org/10.1146/annurev.immunol.23.021704.115830.

Keller, S.M., Vernau, W., Moore, P.F., 2016. Clonality testing in veterinary medicine: a review with diagnostic guidelines. Vet. Pathol. 53, 711–725. https://doi.org/10.1177/0300985815626576.

Keresztes, M.A., Henrich, M., Baloi, P., Gerst, S., Rudnick, J., Langenstein, J., Moritz, A., Bauer, N., 2017. Nonregenerative immune-mediated anemia associated with a diffuse large B-cell lymphoma in a captive jaguar (*Panthera onca*). Vet. Clin. Pathol. 46, 597–604. https://doi.org/10.1111/vcp.12542.

Langerak, A.W., Groenen, P.J.T.A., Brüggemann, M., Beldjord, K., Bellan, C., Bonello, L., Boone, E., Carter, G.I., Catherwood, M., Davi, F., Delfau- Larue, M., Diss, T., Evans, P.A.S., Gameiro, P., Garcia Sanz, R., Gonzalez, D., Grand, D., Hakannson, A., Hummel, M., Liu, H., Lombardia, L., Macintyre, E.A., Milner, B.J., Montes-Moreno, S., Schuuring, E., Spaargaren, M., Hodges, E., van Dongen, J.J.M., 2012. EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. Leukemia 26, 2159–2171. https://doi.org/10.1038/leu.2012.246.

Marker, L., Munson, L., Basson, P.A., Quaokenbush, S., 2003. Multicentric T-cell lymphoma associated with feline leukemia virus infection in a captive Namibian cheetah (*Acinonyx jubatus*). J. Wildl. Dis. 39, 690–695.

Mochizuki, H., Nakamura, K., Sato, H., Goto-Koshino, Y., Sato, M., Takahashi, M., Fujino, Y., Ohno, K., Uchida, K., Nakayama, H., Tsujimoto, H., 2011. Multiplex PCR and Genescan analysis to detect immunoglobulin heavy chain gene rearrangement in feline B-cell neoplasms. Vet. Immunol. Immunopathol. 143, 38–45. https://doi.org/10.1016/j.vetimm.2011.05.030.

Mochizuki, H., Nakamura, K., Sato, H., Goto-Koshino, Y., Sato, M., Takahashi, M., Fukushima, K., Nakashima, K., Fujino, Y., Ohno, K., Nakayama, H., Tsujimoto, H., 2012. GeneScan analysis to detect clonality of T-cell receptor γ gene rearrangement in feline lymphoid neoplasms. Vet. Immunol. Immunopathol. 145, 402–409. https://doi.org/10.1016/j.vetimm.2011.12.015.

Poli, A., Abramo, F., Cavicchio, P., Bandecchi, P., Ghelardi, E., Pistello, M., 1995. Lentivirus infection in an African lion: A Clinical, Pathologic and Virologic Study. J. Wildl. Dis. 31, 70–74.

Rout, E.D., Burnett, R.C., Yoshimoto, J.A., Avery, P.R., Avery, A.C., 2019. Assessment of immunoglobulin heavy chain, immunoglobulin light chain, and T-cell receptor clonality testing in the diagnosis of feline lymphoid neoplasia. Vet. Clin. Pathol. 48 (Suppl. 1), 45–58. https://doi.org/10.1111/vcp.12767.

Stockham, S.L., Scott, M.A., 2008. Introductory concepts. In: Stockham, S.L., Scott, M.A. (Eds.), Fundamentals of Veterinary Clinical Pathology. Blackwell Publishing, Ames, Iowa, USA, pp. 38–39.