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Revisiting lymphocyte clonality testing in feline B-cell lymphoma

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Table of Content

1. Introduction

Cats have the highest incidence of lymphoma compared to any other species. Lymphoma represents 41 % of all malignant feline tumors and is responsible for 90 % of hematopoietic tumors in cats (MacVean et al., 1978; Bienzle and Vernau, 2011; Wolfesberger et al., 2018). Lymphoma is a cancer originating by proliferation of malignant lymphoid cells originating outside the bone marrow and primarily affecting lymph nodes or visceral organs like liver, spleen, kidney, but can affect nearly any organ system (MacVean et al., 1978; Burkhard and Bienzle, 2013).

The most frequent anatomical site in felines is the gastrointestinal tract followed by extranodal, mediastinal and nodal lymphoma (Wolfesberger et al., 2010; Sato et al., 2014). Primary feline intestinal lymphomas are most commonly of T-cell origin, especially in the small intestine and are supposed to derive from the local mucosa-associated lymphoid tissue. In the stomach and large intestine, B-cell lymphomas predominate (Pohlman et al., 2009; Kiupel et al., 2011).

Diagnostic features to identify lymphoma cells are ultrasound-guided, fine-needle aspiration (FNA) cytology, histopathological examination of biopsies, immunohistochemistry, and immunophenotyping by flow cytometry. However, diagnostic dilemmas posed by the quantity and quality of the lymphocytic infiltrates do exist. Major reasons could be inadequate sample size or the presence of non-neoplastic cells. Lymphomas with mixed cellularity or small-cell lymphomas can be missed, whereas severe reactive hyperplasia may be confused with neoplasia (Burkhard and Bienzle, 2013; Hammer et al., 2017).

Lymphomas are the result of a clonal proliferation originating from a single neoplastic B- or T-cell in their stages of lymphoid differentiation; hence, all tumor cells contain a unique clonal immunoglobulin (IG) heavy chain (IGH) or T-cell receptor gamma chain (TCRG) gene rearrangement (van Dongen et al., 2003; Jung et al., 2006; Keller et al., 2016). As clonality is a main characteristic of neoplastic cells, the detection of a clonal nature of a population of lymphocytes in tissue specimens is helpful for the diagnosis of lymphoid neoplasia. Clonality assay technology has been introduced as a complimentary tool to identify a clonal population of lymphocytes and their origin from a distinct B- or T-cell

(Avery et al., 2012; Burkhard and Bienzle, 2013; Unterkreuter et al., 2021). In veterinary medicine, a correct interpretation of PCR derived patterns according to the EuroClonality/BIOMED-2 guidelines for clonality testing in humans is strongly recommended (Langerak et al., 2012; Keller et al., 2016).

In previous studies, the analysis of IGH rearrangement showed a wide range of results, e.g., clonality could be detected in 34-89 % of feline B-cell neoplasms (Werner et al., 2005; Henrich et al., 2009; Mochizuki et al., 2011; Moore et al., 2012; Hammer et al., 2017; Henrich et al. 2018). In contrast, the PCR-based clonality assay to detect clonal TCRG gene rearrangements showed a sensitivity of above 79-90 % (Sato et al. 2011; Weiss et al. 2011; Mochizuki et al., 2012; Gress et al., 2016; Hammer et al. 2017). In B-cell lymphoma, false negative results were found because of altered primer binding sites after somatic hypermutation in the germinal center of a lymphoid follicle due to antigenic stimulation (Moore et al., 2005; Mochizuki et al., 2011; Sato et al. 2011; Weiss et al., 2011; Moore et al., 2012).

In human, incomplete IGH gene rearrangements only involving diversity (D) and joining (J) elements as well as IG light chain gene rearrangements are less affected by somatic hypermutation. Furthermore, during B-cell development in human, IG kappa (κ) light chain (IGK) genes rearrange first. However, if these rearrangements do not lead to a functional κ light chain, inactivation of the IGK allele is controlled by the kappa deleting element (Kde), followed by rearrangement of other IG light chain genes (Van Dongen et al., 2003; Langerak et al., 2011; Ghorbian et al., 2014). Feline B cells express primarily lambda (λ) light chains, suggesting that most of these cells underwent Kde recombination to inactivate the IGK locus (Arun et al., 1996; Cho et al., 1998). Consequently, evaluating Kde and IG lambda light chain (IGL) gene rearrangements should improve the sensitivity of detecting clonality in B-cell neoplasia compared to assessing complete IGH-VDJ rearrangements only (Ghorbian et al., 2014; Rout et al., 2019).

Aim of this study was to optimize the detection of immunoglobulin gene rearrangements in B-cell lymphomas in domestic felines by applying PCR-based clonality testing with novel primer sets targeting complete IGH-VDJ and incomplete IGH-DJ gene rearrangements together with IGK deleting element (Kde) and IGL gene rearrangements (Rout et al., 2019). The underlying hypothesis implies that different primer sets would show different diagnostic efficacy in the detection of molecular clonality. We utilized a

retrospective cohort of feline patients to evaluate these novel primer sets for the detection of clonal lymphocyte populations in B-cell lymphomas in cats. Diagnostic sensitivity, specificity and accuracy together with the positive predictive value (PPV) and the negative predictive value (NPV) were determined.

2. Material and Methods

2.1. Case Selection and Origin of Sample Material

In this retrospective study, 24 domestic feline patients with matched cytological and/ or histopathological review including immunohistochemistry and flow cytometry were assayed for their IGH and TCRG gene rearrangements by clonality testing. Information about the studied animals regarding breed, gender, and age are given in Supp. Table 1. In detail, remnants of biopsy material of 20 feline lymphoma cases, served as study material in the lymphoma group (Table 1, Supp. Table 3). Lymph node material from four cats being euthanized for reasons other than hematopoietic neoplasia and with a confirmed status of a non-neoplastic reactive condition served as controls (Table 1).

Genomic DNA for clonality testing derived from samples of different anatomic locations either collected through a biopsy at the point of diagnosis or taken from dead cats at necropsy (Supp. Table 1). The studied cohort comprised feline patients of the University of Veterinary Medicine Vienna (Vetmed Uni Vienna) and external cases sent in for routine clonality testing at the Clinical Pathology Unit (Vetmed Uni Vienna) between December 2013 and August 2020.

Table 1
Summarizing results of 24 cats enrolled in this study regarding histopathological classification, cytological diagnosis, immunohistochemistry, immunophenotyping, and clonality outcome.

						Primer set A[1]		Primer set B[2]	
Case no	Sample no	Histopathology	IHC	FCM	Cytology	Clonality	Result	Clonality	Result
1	1	n/a	n/a	n/a	Lymphoma	pos: B	TP	n/d	FN
2	2	n/a	n/a	n/a	Lymphoma	n/d	FN	n/d	FN
3	3	DLBCL	BCL	BCL	Lymphoma	n/d	FN	pos: B	TP
4	4	n/a	n/a	n/a	Lymphoma	n/d	FN	pos: B	TP
5	5	n/a	n/a	n/a	Lymphoma	n/d	FN	pos: B	TP
6	6	n/a	n/a	n/a	Lymphoma	n/d	FN	n/d	FN
7	7	n/a	n/a	n/a	Lymphoma	n/d	FN	pos: B	TP
8	8	n/a	n/a	n/a	Lymphoma	n/d	FN	n/d	FN
9	9	DLBCL	BCL	BCL	Lymphoma	n/d	FN	pos: B	TP
10	10	DLBCL	BCL	BCL	Lymphoma	pos: B	TP	n/d	FN
11	11	reactive	reg. distr.	reactive	Reactive hyperplasia	n/d	TN	n/d	TN
12	12	DLBCL	BCL	BCL	Lymphoma	pos: B	TP	n/d	FN
	13				Lymphoma	pos: B	TP	n/d	FN
13	14	n/a	n/a	n/a	Lymphoma	n/d	FN	n/d	FN
14	15	transmural intestinal BCL	n/a	BCL	Lymphoma	n/d	FN	pos: B	TP
	16				Lymphoma	n/d	FN	pos: B	TP
15	17	DLBCL	BCL	n/a	Lymphoma	n/d	FN	n/d	FN
16	18	n/a	n/a	n/a	Lymphoma	pos: B	TP	n/d	FN
17	19	reactive	reg. distr.	reactive	Reactive hyperplasia	n/d	TN	n/d	TN
18	20	DLBCL	BCL	BCL	Lymphoma	pos: B	TP	pos: B	TP
19	21	n/a	n/a	n/a	Lymphoma	pos: B	TP	pos: B	TP
20	22	n/a	n/a	n/a	Lymphoma	pos: B	TP	pos: B	TP
21	23	reactive	reg. distr.	reactive	Reactive hyperplasia	n/d	TN	n/d	TN
22	24	reactive	reg. distr.	reactive	Reactive hyperplasia	n/d	TN	n/d	TN
23	25	DLBCL	BCL	BCL	Lymphoma	pos: B	TP	n/d	FN
	26				Lymphoma	pos: B	TP	n/d	FN
24	27	n/a	n/a	n/a	Lymphoma	pos: B	TP	pos: B	TP

Abbreviations: B-cell lymphoma (BCL); Diffuse large B-cell lymphoma (DLBCL); false negative (FN); Flow cytometry (FCM); Immunohistochemistry (IHC); monoclonal (mc); negative (neg.); not analyzed (n/a); not detected (n/d); oligoclonal (oc); polyclonal (pc); positive for B-cell clonality (pos: B); pseudoclonal (psc); regular distribution (reg. distr.); true negative (TN); true positive (TP).

[1] Mochizuki et al., 2011; Mochizuki et al., 2012.

[2] Rout et al., 2019.

2.2. Diagnostic work-up

Histopathological examination, cytological review, immunohistochemistry (IHC) and immunophenotyping by flow cytometry (FCM) were conducted as previously described (Rütgen et al., 2021). Sample localization and source material for the animals under investigation are summarized in Supp. Table 1.

2.3. DNA extraction and genomic DNA quality control

Total genomic DNA (gDNA) was extracted from single cell suspensions or fine needle aspiration biopsies (FNA) by using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, Vienna, Austria) or the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. In the case of FNA-derived cytology slides, 500 µL of 1 × phosphate buffered saline (1× PBS) and 20 µl Proteinase K (supplied with the kit) were combined in a 2 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany). The majority of the solution was pipetted onto the slide surface; the cell material was scraped off with a tip and quantitatively transferred to the microcentrifuge tube. After adding 200 µL lysis buffer (supplied with the kit), the samples were further processed following the kit's manual.

The concentration and 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 five 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 was PCR-amplified for each sample as described previously (Hammer et al., 2017). Feline lymphoma cell lines MS4 and FT-1 served as positive controls (Mochizuki et al., 2011; Mochizuki et al., 2012).

2.4. Primer sets and PCR protocol for Clonality Testing

For the assessment of B- and T-cell clonality, multiplex PCRs were set up in four different reaction mixtures (tube 1 to 4). For amplification of complete IGH-VDJ gene rearrangements, two specific primers sets were used in comparison (tube 1: Mochizuki et al., 2011; tube 3: Rout et al., 2019). IGH-VDJ specific primers in tube 1 were the same

as the formerly described primer set B (Hammer et al., 2017). As a novelty, primers targeting incomplete IGH-DJ gene rearrangements, kappa deleting element (Kde) and IG lambda light chain (IGL) gene rearrangements were multiplexed in tube 4 (Rout et al., 2019). For the sake of completeness, all samples were assayed for T-cell clonality by applying primers specifically targeting TRG-VJ gene rearrangements (tube 2: Mochizuki et al., 2012). TRG-VJ specific primers in tube 2 were the same as the formerly described primer set B (Hammer et al., 2017). For better reading, the two conventional primer sets targeting complete IGH-VDJ (tube 1) and TRG-VJ gene rearrangements (tube 2) are referred to as 'Primer set A'. Consequently, the two refined multiplex primer sets targeting complete IGH-VDJ (tube 3) and incomplete IGH-DJ, Kde and IGL (tube 4) are referred to as 'Primer set B' throughout this study. Each PCR reaction was carried out in triplicate including positive and negative PCR controls in each PCR run (Hammer et al., 2017). The PCR reactions were run on a T Gradient thermal cycler (Biometra, Göttingen, Germany) with formerly described cycling conditions (Rout et al., 2019).

All PCR reactions were supplemented with 10 µL of DNA Dilution Buffer (Qiagen GmbH, Hilden, Germany) 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 1.6 (Qiagen) (Gress et al., 2016; Hammer et al., 2017; Unterkreuter et al., 2021).

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). A triplicate PCR to evaluate the reproducibility of their respective clonality patterns assayed each sample. In the case of inconsistent triplicates and no observation of a monoclonal peak, the sample was scored as pseudoclonal. Per definition, a tall narrow peak > 3000 in amplitude and at least double the height of the base peaks forming the polyclonal background can be considered as monoclonal. A sample with two distinct peaks matching these criteria was assigned as biclonal, whereas more than two distinct peaks reflect an oligoclonal pattern. Samples with multiple peaks, forming a Gaussian distribution within the expected size

range were considered polyclonal (Keller et al., 2016). Definite single peaks surrounded by a polyclonal setting were defined as monoclonal with a polyclonal background (Hammer et al., 2017). IGH-DJ, Kde and IGL rearrangements have smaller junctional areas compared with IGH-VDJ rearrangements and their polyclonal distributions were narrower and less Gaussian in shape compared with the IGH-VDJ rearrangements (Rout et al., 2019).

2.6. Statistical evaluation

Histopathology and cytology served as gold standard for the diagnosis of reactive hyperplasia or lymphoma so that true positives, true negatives, false positive and false negative clonality testing results could be identified. Diagnostic sensitivity, specificity, accuracy, positive and negative predictive values were calculated by a Bayes' diagram (Stockham and Scott, 2008).

3. Results and discussion

3.1. Diagnostic work up

Specimens were subjected to cytological and histopathological review, IHC together with FCM (Table 1) as well as comparative B-cell clonality testing (Supp. Table 2, Supp. Fig. 1A). Histopathological findings were available for twelve cat patients, seven with Diffuse Large B-cell Lymphoma (DLBCL) (case 3, 9, 10, 12, 15, 18, and 23) and one intestinal BCL (case 14), where no further classifying was done together with four reactive cases (11, 17, 21, and 22). IHC was done on eleven patients, seven were positive for the studied B-cell markers (case 3, 9, 10, 12, 15, 18, and 23) and the four reactive cases (11, 17, 21, and 22) showed a regular distribution of B and T lymphocytes. Eleven patients were subjected to FCM. In seven cases (3, 9, 10, 12, 14, 18, and 23), a dominant B-cell marker expression was found and the four reactive cases (11, 17, 21, and 22) were characterized by a mixed marker expression of analyzed lymphocytes. Cytology was available for all 24 cases, 20 of them showing lymphoma and four reactive hyperplasia, respectively. In summary, ten cat patients were characterized by a complete diagnostic work up, being comprised of six lymphoma (3, 9, 10, 12, 18, and 23) and four lymphoid reactive hyperplastic cases (11, 17, 21, and 22) (Table 1).

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

Twenty-seven gDNA samples originated from 24 domestic cats and their age at date of biopsy sampling ranged from one to 16 years (median 9 years). Among these 24 cats, eleven were castrated males and 13 spayed females. With respect to breeds, the studied cohort included 17 European Shorthair cats, one Persian, one Abyssinian, one Siamese, one Russian blue, and three cats with unknown breed. For three cat patients, the gDNAs derived from two different locations, intestinal wall and abdominal lymph node (case no. 12, sample no. 12 and 13), ileocecal area and mass on the outside of the intestine (case no. 14, sample no. 15 and 16), stomach and jejunum (case no. 23, sample no. 25 and 26) (Supp. Table 1).

Depending on the cellularity of the samples, the volume of the isolated gDNA varied from 65 to 150 µL and the concentration ranged from 6.8 to 409.1 ng/µL with a mean value of

96.85 ng/µL (Supp. Table 2). The 260/280 ratio exhibited a maximum of 2.08, a minimum of 1.37 and a mean value of 1.93. In total, 27 gDNA samples being obtained from 24 animals were successfully amplified for the 189 bp fragment of the feline androgen receptor gene (data not shown).

3.3. Consistency of clonality patterns with histopathological and cytological evidence

The clonality results for primer sets A and B are summarized in Table 1, Supp. Table 2, Supp. Fig. 1A and Supp. Fig.2. In total, twelve out of 20 histopathological and/or cytological confirmed lymphoma cases returned either polyclonal or pseudoclonal after assessment with primer set A targeting complete IGH-VDJ gene rearrangements (tube 1) and were therefore assigned as false negative. The application of primer set B confirmed seven false negative cases as true positive (case 3, 4, 5, 7, 9 and 14). To better illustrate the performance of primer set B targeting complete IGH-VDJ (tube 3) as well as incomplete IGH-DJ, Kde and IGL (tube 4), three representative cases (7, 4 and 14) are presented in Fig. 1. However, in five out of twelve cases, primer set B failed to confirm false negative cases as true positive (case 2, 6, 8, 13, and 15). In case 2, 6, and 15, PCR reactions for IGH-VDJ (tube 1) showed either a pseudoclonal or a polyclonal pattern. For cases 2, 6 and 15, tube 3 returned polyclonal patterns but two of them exhibiting solitary peaks at 145 bp (case 2) and 136 bp (case 15) that are too small in height to be considered as monoclonal. In case 2 and 6, PCR for IGH-DJ, Kde and IGL (tube 4) revealed a polyclonal pattern, with the exception of a negative Kde PCR for case 6. In contrast, in case 15, those PCR reactions (tube 4) were negative. Unfortunately, PCR reactions for IGH-VDJ (tube 3) are missing in case 8 and 13 due to lack of genomic DNA. Primer set A detected B-cell clonality in twelve out of 20 true positive lymphoma cases. Therefore, it was obvious to at least confirm these findings by using primer set B. Surprisingly, PCR reactions being set up with primer set B corroborated only four out of these twelve true positive cases (18, 19, 20, and 24), whereas the remaining cases returned polyclonal, hence false negative (Fig. 2). On the other hand, all four histopathological and cytological confirmed reactive (non-neoplastic), hence true negative cases (11, 17, 21, and 22) returned polyclonal after assessment with primer set A and B targeting complete IGH-VDJ gene rearrangements (tube 1 and 3) as well as incomplete IGH-DJ, Kde and IGL (tube 4). To better illustrate successful matching of

both primer sets, three representative cases (18, 19 and 17) are presented in Fig. 3. The joint confirmatory power of both primer sets resulted in 18 true positive, four true negative, and five false negative clonality results (Supp. Fig. 1A and B).

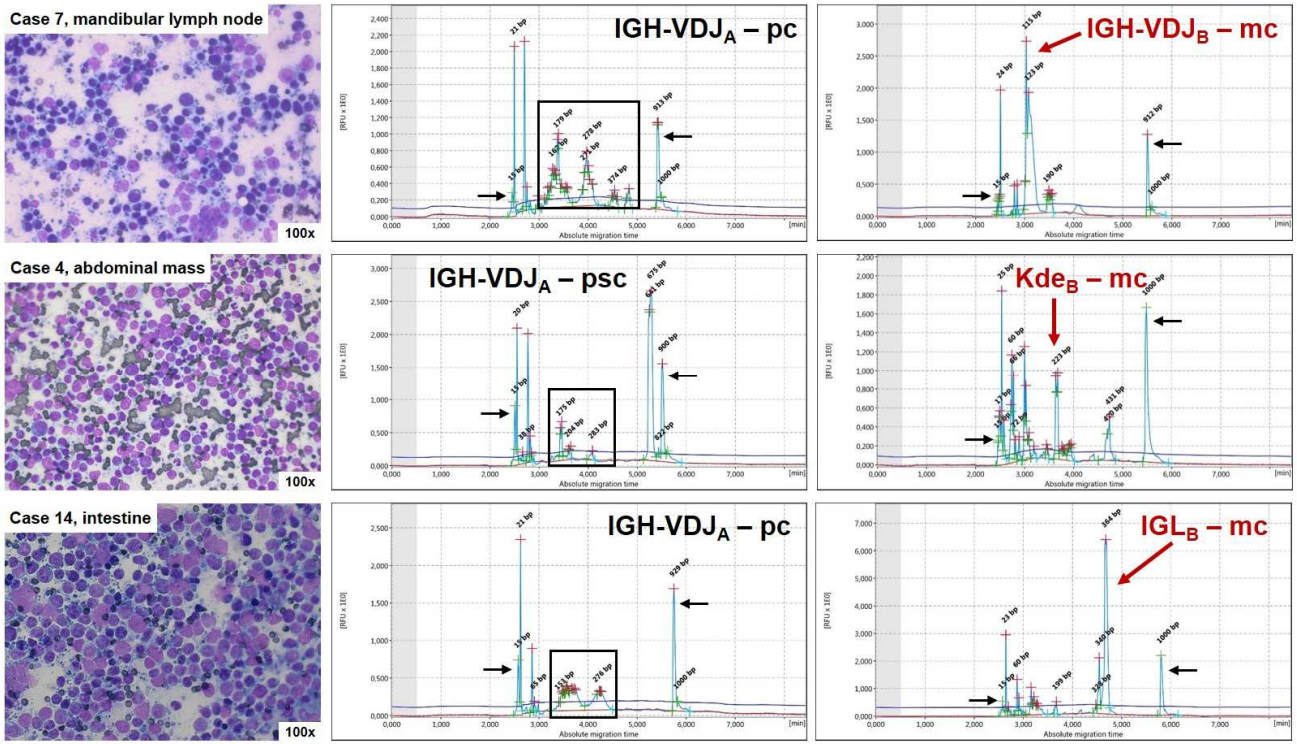

Fig. 1. Cytology images (first column) and traces from clonality assays (column 2 and 3) are shown for three representative true positive feline lymphoma patients (case 7, 4 and 14). In the clonality assay, IGH-VDJ (A) (primer set A: Mochizuki et al., 2011), IGH-VDJ (B), Kde (B) or IGL (B) (primer set B: Rout et al., 2019) indicate B-cell clonality. Three different localizations, mandibular lymph node, abdominal mass and intestine of the three cats are depicted. Magnifications for cytology slides are indicated in the figures. In the analyzed samples, the result of clonality testing is displayed along with the raw signal. The peaks of the alignment marker are indicated with black arrows: 15 bp and 1000 bp. The presence and size of fragments was accurately determined using QIAxcel ScreenGel Software 1.6 (Qiagen). Abbreviations: diversity (D); immunoglobulin heavy chain (IGH); immunoglobulin lambda light chain (IGL); Kappa-deleting element (Kde); joining (J); monoclonal (mc); polyclonal (pc); pseudoclonal (psc); variable (V).

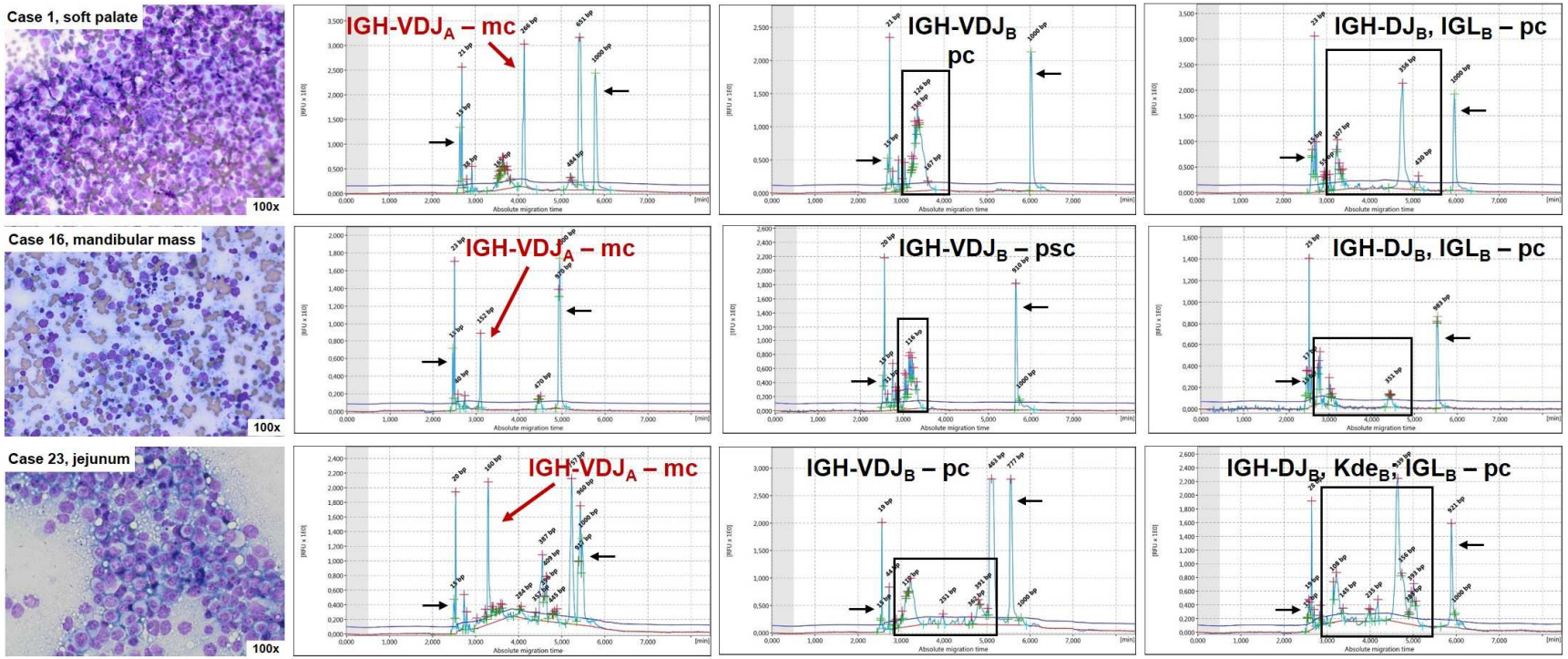

Fig. 2. Cytology images (first column) and traces from clonality assays (column 2 to 4) are shown for three representative false negative feline lymphoma patients (case 1, 16 and 23). In the clonality assay, IGH-VDJ (A) (primer set A: Mochizuki et al., 2011), IGH-VDJ (B), IGH-DJ (B), Kde (B) or IGL (B) (primer set B: Rout et al., 2019) indicate B-cell clonality. Three different localizations, soft palate, mandibular mass and jejunum of the three cats are depicted. Magnifications for cytology slides are indicated in the figures. In the analyzed samples, the result of clonality testing is displayed along with the raw signal. The peaks of the alignment marker are indicated with black arrows: 15 bp and 1000 bp. The presence and size of fragments was accurately determined using QIAxcel ScreenGel Software 1.6 (Qiagen). Abbreviations: diversity (D); immunoglobulin heavy chain (IGH); immunoglobulin lambda light chain (IGL); Kappa-deleting element (Kde); joining (J); monoclonal (mc); polyclonal (pc); pseudoclonal (psc); variable (V).

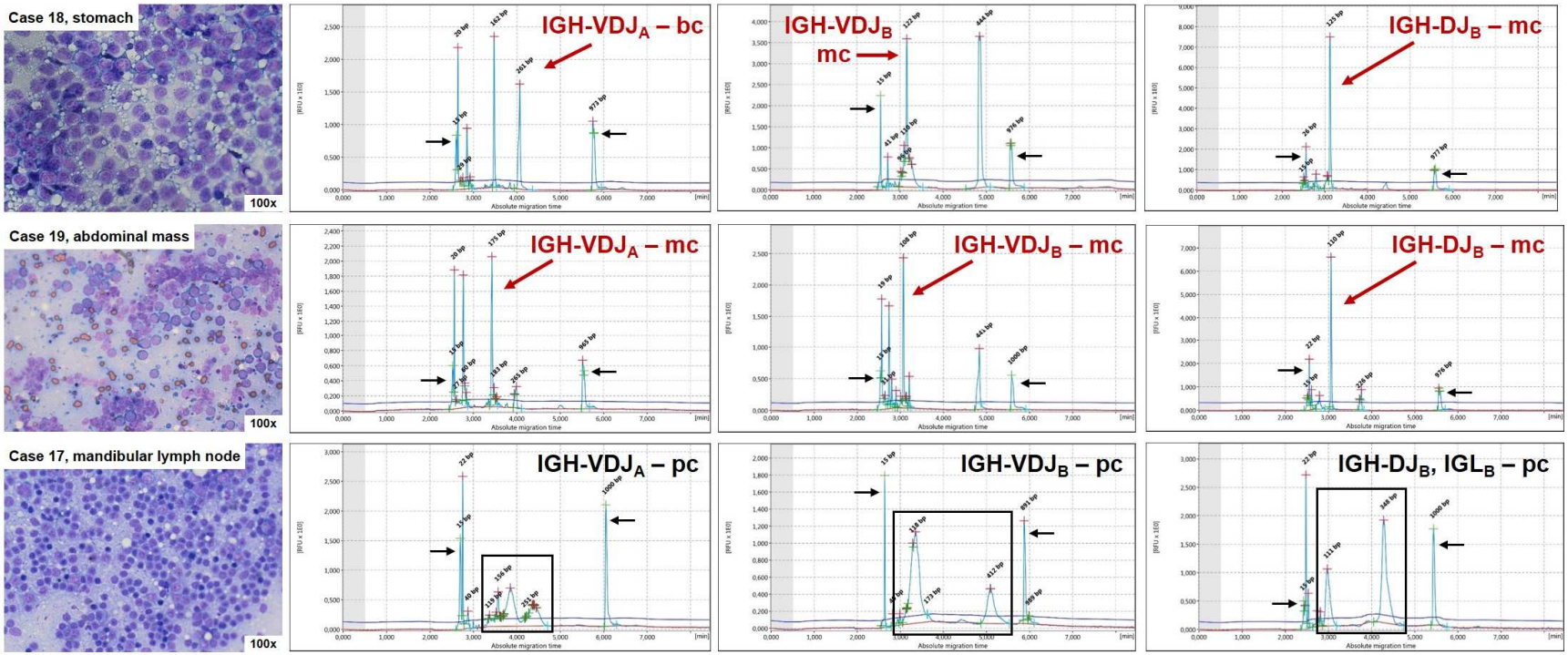

Fig. 3. Cytology images (first column) and traces from clonality assays (column 2 to 4) are shown for two representative feline lymphoma patients (case 18 and 19) and one non-neoplastic patient (case 17) demonstrating the uniformity of primer sets A and B. In the clonality assay, IGH-VDJ (A) (primer set A: Mochizuki et al., 2011), IGH-VDJ (B), IGH-DJ (B) or IGL (B) (primer set B: Rout et al., 2019) indicate B-cell clonality. Three different localizations, stomach, abdominal mass and mandibular lymph node of the three cats are depicted. Magnifications for cytology slides are indicated in the figures. In the analyzed samples, the result of clonality testing is displayed along with the raw signal. The peaks of the alignment marker are indicated with black arrows: 15 bp and 1000 bp. The presence and size of fragments was accurately determined using QIAxcel ScreenGel Software 1.6 (Qiagen). Abbreviations: biclonal (bc); diversity (D); immunoglobulin heavy chain (IGH); immunoglobulin lambda light chain (IGL); joining (J); monoclonal (mc); polyclonal (pc); variable (V).

3.4. Revisiting sensitivity and specificity of used primer sets

Previous studies have designed primers directed against the most common IGHV-3 subgroups, the less common IGHV1 subgroups, the two most commonly rearranged IGHJ genes and degenerate primers for the variability in the remaining IGHJ genes (Werner et al., 2005; Henrich et al., 2009, Mochizuki et al. 2011). The sensitivity for detecting a clonal B-cell receptor in feline lymphomas varies from 34 to 89 % (Werner et al., 2005; Henrich et al., 2009; Mochizuki et al., 2011; Moore et al., 2012; Hammer et al., 2017; Henrich et al. 2018). The most common cause for false negative clonality results is the failure to generate a clonal peak mostly because one or both primers fail to anneal to their target sequence that potentially was altered during somatic hypermutation (Van Dongen et al., 2003; Keller et al., 2016; Rout et al. 2019). In human medicine, predominantly DLBCL accumulate large numbers of somatic mutations (Van Dongen et al., 2003). To improve the assay's sensitivity, novel primer sets have been developed to assess clonality of alternative IG rearrangements by targeting incomplete IGH-DJ gene rearrangements together with IGK deleting element (Kde) and IG lambda light chain (IGL) gene rearrangements (Rout et al., 2019).

Rout (2019) reported to detect 32 % incomplete IGH-DJ, 29 % Kde and 26 % IGL rearrangements (Rout et al., 2019). Among the studied cohort of 24 feline patients, we also found 30 % IGH-DJ recombination, but only 8 % Kde or IGL rearrangements. However, without using Kde primers we would have missed two true positive feline B-cell lymphomas, namely cases 4 and 9 (Fig. 1). In contrast, IGL positivity of cases 14 and 20 was corroborated either by incomplete or complete IGH clonality (Supp. Table 1, Supp. Fig. 1A).

Compared to previous studies, diagnostic sensitivity and specificity of primer set B were 48 % and 100 % (Hammer et al., 2017; Rout et al., 2019). Overall, diagnostic accuracy was 56 %, positive predictive value 100 % and negative predictive value 25 % (Supp. Fig. 1B). Notably, none of the primer sets was superior; hence, we recommend a joint application of the herein tested primer sets in routine diagnostics. In conclusion, combined application of primer set A and B increased diagnostic sensitivity to 78 %, diagnostic accuracy to 81 % and negative predictive value to 44 % (Supp. Fig. 1B). However, a more in-depth-evaluation of the dynamic of assay specific parameters in

dependency on primer set usage requires prospective studies on larger cohorts of feline patients.

4. Conclusion

In general, we can say that in our study the different primer sets detect clonality in different samples, without a superiority of one of the primer-set. In fact, diagnostic sensitivity, specificity, accuracy, positive predictive value were significantly increased by considering both primer-set, only the negative predictive value underwent a slight impairment. We suggest a combined application of primer set A and B to reduce false negative results in routine diagnostics for lymphoma. It is clear that for a more-in-depth evaluation of the dynamic of assay specific parameters in dependency on primer set usage, further prospective studies on larger cohorts of feline patients are required. Furthermore, there are still false negative samples, which cannot be detected. Thus, there is still a need of further primers directed against other gene loci to detect clonality in these cases.

As a result, it is important that one should not forget to consider clinical conditions and all other diagnostic findings and to not rely only on clonality testing to keep aware of false positive and false negative results and to enable making an accurate diagnosis.

5. Zusammenfassung

Die Differenzierung zwischen ausgereiften Lymphozyten Populationen und neoplastischen Vorgängen, bei denen kleine Lymphozyten betroffen sind (kleinzelliges Lymphom), kann sich anhand von zytologischen Untersuchungen als schwierig gestalten. In diesen Fällen muss auf zusätzliche diagnostische Mittel zurückgegriffen werden, wie die PARR (PCR for antigen receptor rearrangements) bzw. die PCR-basierte Immunoglobulin (IG) und T-Zell-Rezeptor Klonalitätsprüfung. Auch wenn immer mehr Primer entwickelt werden, um alle potentielle IG Gen-Umgruppierungen zu erfassen, kommt es immer noch zu falsch negativen und falsch positiven Ergebnissen in felinen B-Zell Lymphomen. In dieser retrospektiven Studie haben wir die diagnostische Sensitivität und Spezifität von einem neu entwickelten PCR Assay für die Routine-Diagnostik von B-Zell-Klonalität beurteilt. Für diesen Zweck wurden 24 feline Patienten vergleichenden Klonalitätsprüfungen mit verschiedenen Primer-Sets unterzogen. Feline Lymphoma-Zelllinien und bestätigtes Patientenmaterial dienten als Positivkontrollen. Im Vergleich zu früheren Studien hat dieser neu entwickelte multiplex PCR Assay positive Auswirkungen auf die diagnostische Sensitivität, Spezifität, Präzision, sowie den positiven prädiktiven Wert, begleitet von einer schwachen Verschlechterung des negativen prädiktiven Werts. Keiner von den verschiedenen Primer-Sets hat sich als überlegen dargestellt, sodass wir in der Routinediagnostik eine kombinierte Anwendung der hier getesteten Primer-Sets empfehlen. Trotzdem benötigt eine tiefgründige Evaluierung der Dynamik der Assay-spezifischen Parameter in Abhängigkeit des verwendeten Primer-Sets prospektive Studien mit größeren Kohorten von felinen Patienten.

6. Summary

Differentiation between resident mature lymphocyte populations and small-cell lymphoma cannot be made by cytological review alone and remains challenging in histopathological review. These cases warrant application of complementary tools like PCR-based immunoglobulin (IG) and T-cell receptor (TCR) clonality testing for confirmation. Although primer coverage of potential IG gene rearrangements in feline B-cell neoplasms constantly improves, the possibility of false negative and false positive test results still poses a problem. In this retrospective study, we assessed diagnostic sensitivity and specificity of a novel developed multiplex PCR assay for routine diagnosis of B-cell clonality. Therefore, 24 feline patients were subjected to comparative clonality testing by using different primer sets. Feline lymphoma cell lines and confirmed patient material served as positive control. Compared to previous studies, this novel developed multiplex PCR assay showed positive effects on diagnostic sensitivity, specificity, accuracy, and positive predictive value accompanied by a slight impairment of negative predictive value. Notably, none of the primer sets was superior; hence, we recommend the combined application of the herein tested primer sets in routine diagnostics. However, a more in-depth-evaluation of the dynamic of assay specific parameters in dependency on primer set usage requires prospective studies on larger cohorts of feline patients.

7. Abbreviations

IG = immunoglobulin
IGH = immunoglobulin heavy chain
TCRG = T-cell receptor gamma chain
PCR = poly chain reaction
V = variety
D = diversity
J = joining
IGL = immunoglobuline light chain
IGK = Immunoglobuline light chain kappa
Kde = kappa deleting element
IGL = immunoglobuline llight chain lambda
IGH-VDJ = immunoglobuline heavy chain- variety-diversity-joining element
PPV = positive predictive value
NPV = negative predictive value
IHC = immunohistochemistry
FCM = flow cytometry
FNA = fine needle aspiration
BCL = B-cell lymphoma
DLBCL = Diffuse Large B-cell Lymphoma
PARR = PCR for antigen receptor rearrangements
mc = monoclonal
psc = pseudoclonal
pc = polyclonal
bc = biclonal
oc = oligoclonal
JP = Japan
US = United States
no = number
neg = negative

n/a = not analysed

n/d = not detected

pos. B.= positive for B-cell clonality

reg. distr. = regular distribution

TN = true negative

TP = true positive

ESH = European Short Hair

fc = female castrated

mc = male castrated

uk = unknown

ln = lymph node

FeLV = Feline leukemia virus

FIV = Feline Immunodeficiency virus

CBC = complete blood count

CT = computed tomography

MRI = magnetic resonance imaging

OS = object slide

scs = single cell suspension

IFT = intravenous fluid therapy

COP = Cyclophosphamid (250-300 mg/m2 p.o. every 21 d, Endoxan®, Baxter GmbH, Illinois, USA)- Vincristine (0,75 mg/m2 i.v. every 7 d during 4 weeks, then every 21 d, Oncovin®, Stada GmbH, Bad vilbel, Germany)- Prednisolonacetat 40 mg/m^2(1 mg/kg p.o. SID during 4 weeks, then BID, Prednisolonacetat, Prednicortone® 5 mg pills, Le Vet Pharma B.V., Oudewater, Netherlands) (Day 2012) or (3 mg/kg SID p.o., Nycomed® 25 mg-pills, Takeda Austria GmbH, Vienna, Austria) or Methylprednisolone acetate 40 mg/ml plus Lidocaine hydrochloride 10 mg/ml (20 mg s.c., Depo-Medrol® with Lidocaine injection, Pfizer Limited, Sandwich, Kent, UK) (Day, 2012)

COP plus Cytarabin (800 mg/m2 single injection, Alexan® Cytosar-500 mg/ml, Cikarang Bekasi, Indonesia)

LSA = Lymphosarcoma

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9. List of Tables and Figures

Figure 2. Cytology images (first column) and traces from clonality assays (column 2 to 4) are shown for three representative false negative feline lymphoma patients (case 1, 16 and 23). In the clonality assay, IGH-VDJ (A) (primer set A: Mochizuki et al., 2011), IGH-VDJ (B), IGH-DJ (B), Kde (B) or IGL (B) (primer set B: Rout et al., 2019) indicate B-cell clonality. Three different localizations, soft palate, mandibular mass and jejunum of the three cats are depicted. Magnifications for cytology slides are indicated in the figures. In the analyzed samples, the result of clonality testing is displayed along with the raw signal. The peaks of the alignment marker are indicated with black arrows: 15 bp and 1000 bp. The presence and size of fragments was accurately determined using QIAxcel ScreenGel Software 1.6 (Qiagen). Abbreviations: diversity (D); immunoglobulin heavy chain (IGH); immunoglobulin lambda light chain (IGL); Kappa-deleting element (Kde); joining (J); monoclonal (mc); polyclonal (pc); pseudoclonal (psc); variable (V)…… ……13

Figure 3. Cytology images (first column) and traces from clonality assays (column 2 to 4) are shown for two representative feline lymphoma patients (case 18 and 19) and one non-neoplastic patient (case 17) demonstrating the uniformity of primer sets A and B. In the clonality assay, IGH-VDJ (A) (primer set A: Mochizuki et al., 2011), IGH-VDJ (B), IGH-DJ (B) or IGL (B) (primer set B: Rout et al., 2019) indicate B-cell clonality. Three different localizations, stomach, abdominal mass and mandibular lymph node of the three cats are depicted. Magnifications for cytology slides are indicated in the figures. In the analyzed samples, the result of clonality testing is displayed along with the raw signal. The peaks of the alignment marker are indicated with black arrows: 15 bp and 1000 bp. The presence and size of fragments was accurately determined using QIAxcel ScreenGel Software 1.6 (Qiagen). Abbreviations: biclonal (bc); diversity (D); immunoglobulin heavy chain (IGH); immunoglobulin lambda light chain (IGL); joining (J); monoclonal (mc); polyclonal (pc); variable (V)…… ……14

10. Appendix

Supplementary Table 1
Characteristics of 24 cats enrolled in this study.

Case no	Sample no	Breed	Gender	Age[1]	Localization	Source material
1	1	ESH	fc	7	mass of the soft palate	4x OS FNA
2	2	ESH	mc	4	gastric wall	4x OS FNA
3	3	Persian	mc	8	stomach	5 x 10^6 cells (scs)
4	4	ESH	fc	16	abdominal mass	4x OS FNA
5	5	ESH	mc	9	submandibular, praescapular, popliteal lymph nodes	1x OS FNA
6	6	ESH	fc	1	mass in front of shoulder joint	3x OS FNA
7	7	ESH	fc	11	mandibular lymph node	2x OS FNA
8	8	ESH	fc	6	stomach	3x OS FNA
9	9	Abyssinian	fc	7	solid material ileocaecal trap	OS Paraffine
10	10	ESH	fc	13	stomach and gastric lymph nodes	5 x 10^6 cells (scs)
11	11	ESH	mc	10	popliteal and mandibular lymph nodes	5 x 10^6 cells (scs)
12	12	Siamese	fc	10	intestine wall	5 x 10^6 cells (scs)
	13				abdominal lymph node	5 x 10^6 cells (scs)
13	14	n/a	mc	15	abdominal lymph node	5x OS FNA
14	15	ESH	mc	12	ileocaecal area	5 x 10^6 cells (scs)
	16				mass on the outside of the intestine	5 x 10^6 cells (scs)
15	17	ESH	mc	7	intestine and mass on the outside of intestine	5 x 10^6 cells (scs)
16	18	ESH	fc	6	right mandibular mass	5x OS
17	19	ESH	fc	11	Ln mandibularis	5 x 10^6 cells (scs)
18	20	ESH	fc	8	stomach	5 x 10^6 cells (scs)
19	21	n/a	mc	4	abdominal mass	1x OS FNA
20	22	n/a	mc	13	intestine	3x OS FNA
21	23	ESH	mc	3	mandibular lymph node	5 x 10^6 cells (scs)
22	24	ESH	mc	n/a	popliteal lymph node	5 x 10^6 cells (scs)
23	25	Russian blue	fc	11	stomach	5 x 10^6 cells (scs)
	26				jejunum	5 x 10^6 cells (scs)
24	27	ESH	fc	11	mass or lymph node	2x FNA

Abbreviations: European short hair (ESH); female spayed (fs); Fine needle aspiration (FNA); not available (n/a); male castrated (mc); object slides (OS); single cell suspension (scs).

[1] Age is given in years.

Supplementary Table 2

DNA quality assessment and Gene Scanning results of 24 domestic feline individuals (equal to 27 genomic DNA samples) analyzed in this study.

			genomic DNA		Tube 1	Tube 2	Primer set A[1,2]		Tube 3	Tube 4			Primer set B[3]	
Case no	Sample no	Date	[ng/µl]	260/280	IGH-VDJ	TRG/J1-J3	Clonality	Result	IGH-VDJ	IGH-DJ	Kde	IGL	Clonality	Result
1	1	15.01.2015	67.00	1.92	mc	pc	pos: B	TP	pc	pc	neg.	pc	n/d	FN
2	2	10.03.2015	120.00	1.90	psc	pc	n/d	FN	pc	pc	pc	pc	n/d	FN
3	3	23.09.2016	96.85	1.95	pc	pc	n/d	FN	mc + pc bg	mc	neg.	pc	pos: B	TP
4	4	16.01.2017	161.30	2.02	psc	psc	n/d	FN	pc	pc	mc + pc bg	pc	pos: B	TP
5	5	22.05.2017	145.25	1.90	pc	pc	n/d	FN	mc	mc + pc bg	neg.	pc	pos: B	TP
6	6	21.06.2017	155.70	1.88	psc	pc	n/d	FN	pc	pc	neg.	pc	n/d	FN
7	7	19.09.2017	131.15	1.93	pc	pc	n/d	FN	mc + pc bg	pc	neg.	pc	pos: B	TP
8	8	15.02.2018	36.00	1.95	psc	psc	n/d	FN	n/a	pc	neg.	pc	n/d	FN
9	9	21.02.2018	409.10	1.99	pc	pc	n/d	FN	pc	pc	mc + pc bg	pc	pos: B	TP
10	10	22.03.2018	97.70	1.94	mc	pc	pos: B	TP	pc	pc	pc	pc	n/d	FN
11	11	26.06.2018	6.80	1.37	pc	pc	n/d	TN	pc	psc	neg.	pc	n/d	TN
12	12	09.10.2018	80.40	1.92	mc	pc	pos: B	TP	pc	pc	pc	pc	n/d	FN
	13		85.55	1.92	mc	pc	pos: B	TP	pc	pc	pc	pc	n/d	FN
13	14	05.11.2018	28.80	1.92	pc	pc	n/d	FN	n/a	pc	neg.	pc	n/d	FN
14	15	14.12.2018	91.60	1.92	pc	pc	n/d	FN	pc	mc	neg.	psc	pos: B	TP
	16		92.95	1.93	psc	pc	n/d	FN	pc	mc	neg.	mc	pos: B	TP
15	17	05.04.2019	156.40	1.93	pc	pc	n/d	FN	pc	neg.	neg.	neg.	n/d	FN
16	18	08.04.2019	118.35	2.03	mc	pc	pos: B	TP	pc	psc	neg.	pc	n/d	FN
17	19	22.05.2019	209.10	1.87	pc	pc	n/d	TN	pc	pc	neg.	pc	n/d	TN
18	20	09.07.2019	167.55	1.90	bc	psc	pos: B	TP	mc + pc bg	mc	neg.	pc	pos: B	TP
19	21	22.07.2019	214.10	2.08	mc	pc	pos: B	TP	mc	mc	neg.	pc	pos: B	TP
20	22	09.09.2019	74.90	2.02	bc + pc bg	pc	pos: B	TP	mc + pc bg	pc	neg.	mc	pos: B	TP
21	23	23/07/2020	114.80	1.97	pc	pc	n/d	TN	pc	pc	neg.	pc	n/d	TN
22	24	27/07/2020	227.40	1.87	pc	pc	n/d	TN	pc	pc	neg.	pc	n/d	TN
23	25	28.05.2020	51.40	1.96	mc	pc	pos: B	TP	pc	pc	pc	psc	n/d	FN
	26		88.50	1.93	mc	pc	pos: B	TP	pc	pc	pc	pc	n/d	FN
24	27	26.08.2020	128.10	2.05	bc	psc	pos: B	TP	mc	mc	pc	neg.	pos: B	TP

[1] Mochizuki et al., Vet Immunol Immunopathol. 143 (2011) 38-45.
[2] Mochizuki et al., Vet Immunol Immunopathol. 145 (2012) 402-409.
[3] Rout et al., Vet Clin Path. 48, Suppl 1 (2019) 45-58.

mc = monoclonal
pc = polyclonal
psc = pseudoclonal
bg = background
neg. = negative
pos: B = positive for B-cell clonality

n/d= not detected
n/a = not analyzed

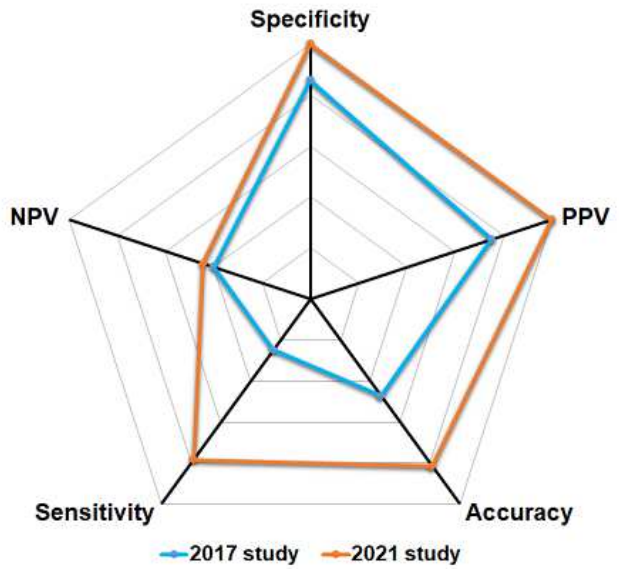
TP = true positive
FN = false negative
TN = true negative

A

		Primer set A[1]		Primer set B[2]			
		Tube 1	Tube 2	Tube 3	Tube 4		
Case no	Sample no	IGH-VDJ	TRG/J1-J3	IGH-VDJ	IGH-DJ	Kde	IGL
1	1	mc, bc	pc	pc	pc	neg.	pc
2	2	psc	pc	pc	pc	pc	pc
3	3	pc	pc	mc, bc	mc, bc	neg.	pc
4	4	psc	psc	pc	pc	mc, bc	pc
5	5	pc	pc	mc, bc	mc, bc	neg.	pc
6	6	psc	pc	pc	pc	neg.	pc
7	7	pc	pc	mc, bc	pc	neg.	pc
8	8	psc	psc	neg.	pc	neg.	pc
9	9	pc	pc	pc	pc	mc, bc	pc
10	10	mc, bc	pc	pc	pc	pc	pc
11	11	pc	pc	pc	pc	neg.	pc
12	12	mc, bc	pc	pc	pc	pc	pc
	13	mc, bc	pc	pc	pc	pc	pc
13	14	pc	pc	neg.	pc	neg.	pc
14	15	pc	pc	pc	mc, bc	neg.	pc
	16	pc	pc	pc	mc, bc	neg.	mc, bc
15	17	pc	pc	pc	neg.	neg.	neg.
16	18	mc, bc	pc	psc	pc	neg.	pc
17	19	pc	pc	pc	pc	neg.	pc
18	20	mc, bc	psc	mc, bc	mc, bc	neg.	pc
19	21	mc, bc	pc	mc, bc	mc, bc	neg.	pc
20	22	mc, bc	pc	mc, bc	pc	neg.	mc, bc
21	23	pc	pc	pc	pc	neg.	pc
22	24	pc	pc	pc	pc	neg.	pc
23	25	mc, bc	pc	pc	pc	pc	pc
	26	mc, bc	pc	pc	pc	pc	pc
24	27	mc, bc	psc	mc, bc	mc, bc	pc	neg.

mc, bc
pc
psc
neg.
no PCR

B



	2017 study	2021 study
Specificity	85.71	100.00
PPV	75.00	100.00
Accuracy	47.37	81.50
Sensitivity	25.00	78.30
NPV	40.00	44.40

Supplementary Figure 1. For 20 histopathological or cytological confirmed feline B-cell lymphoma cases and 4 non-neoplastic controls, the outcome of the PCR-based clonality testing is shown. The heat map (A) summarizes the detection of clonality in 24 feline patients. The results for the primers sets A and B are illustrated by the given color-coding. Orange boxes: Monoclonal or biclonal results. Blue and purple boxes: Polyclonal and pseudoclonal results. Grey and light grey boxes: Negative or missing PCR reactions, latter due to lack of sample material. Yellow boxes: Feline samples being illustrated in Figure 1, 2 and 3. (B) Assay-specific parameters describe the specificity, accuracy and sensitivity of clonality testing to detect clonal rearrangements in B-cell lymphomas and reactive source material. Abbreviations: biclonal (bc); diversity (D); immunoglobulin heavy chain (IGH); immunoglobulin light chain (IGL); Kappa-deleting element (Kde); Japan (JP); joining (J); monoclonal (mc); negative (neg); negative predictive value (NPV); number (no); polyclonal (pc); positive predictive value (PPV); pseudoclonal (psc); United States (US); variable (V); [1] Mochizuki et al., 2011; Mochizuki et al., 2012. [2] Rout et al., 2019; 2017 study: Hammer et al., 2017.

Supplementary table 3: Full case history of 24 domestic feline individuals analyzed in this study

case no	breed	gender	age[1]	date of presentation	symptoms
1	ESH	fc	7	12.01.15	dyspnoa, chronic rhinitis through hyperplasia of retropharyngeal lymph nodes and palatinum, pneumomediastinum
2	ESH	mc	4	10.03.15	diarrhea, vomiting, blood vomiting, alopecia
3	Persian	mc	8	23.09.16	inappetence, anorexia, vomiting, epileptical seizure, dyspnoa
4	ESH	fc	16	16.01.17	uk
5	ESH	mc	9	22.05.17	uk
6	ESH	fc	1	21.06.17	uk
7	ESH	fc	11	19.09.17	inappetence, vomiting
8	ESH	fc	6	15.02.18	uk
9	Abyssinian	fc	7	21.02.18	inappetence, diarrhea, abdomina lump
10	ESH	fc	13	22.03.18	vomiting, only eats fluid food
11	ESH	mc	10	26.06.18	anemia, cachexia, dyspnoea, abdominal lump, difficulties to swallow
12	Siamese	fc	10	08.10.18	weight loss, reduced general condition, diarrhea, vomiting, abdominal mass
13	uk	mc	15	05.11.18	reduced general condition
14	ESH	mc	12	14.12.18	hematoma at caudal abdomen and at perianal region, impaction, inappetence, vomiting, polydypsia
15	ESH	mc	7	05.04.19	vomiting, polydypsia, weight loss
16	ESH	fc	6	08.04.19	right mandibular mass
17	ESH	fc	11	22.05.19	anorexia, icterus, weight loss
18	ESH	fc	8	09.07.19	anorexia, vomiting, abdominal lump, diarrhea
19	uk	mc	4	22.07.19	uk
20	uk	mc	13	09.09.19	uk
21	ESH	mc	3	23.07.20	uk
22	ESH	mc	uk	27.07.20	uk
23	Russian blue	fc	11	28.05.20	polydypsia, headshaking, vomiting, bruxism
24	ESH	fc	11	26.08.20	uk

no = number
ESH = European Short Hair
fc = femlale castrated
mc = male castrated
uk = unknown
ln = lymph node
FeLV = Feline leukemia virus
FIV = Feline Immunodeficiency Virus
CBC = complete blood count
CT = computed tomography
MRI = magnetic resonance imaging
OS = object slides
FNA = Fine needle aspiration
scs = single cell suspension
IHC = immunohistochemistry

FCM = flow cytometry
BCL = B-cell lymphoma
DLBCL = Diffuse large B-cell lymphoma
IFT = intraveneous fluid therapy
LSA = lymphosarcoma

* Valli et al., 2011
[1] Age is given in years.

COP = Cyclophosphamid (250-300 mg/m2 p.o. every 21 d, Endoxan®, Baxter GmbH, Illinois, USA)- Vincristine (0,75 mg/m2 i.v. every 7 d during 4 weeks, then every 21 d, Oncovin®, Stada GmbH, Bad vilbel, Germany)- Prednisolonacetat 40 mg/m2(1 mg/kg p.o. SID during 4 weeks, then BID, Prednisolonacetat, Prednicortone® 5 mg pills, Le Vet Pharma B.V., Oudewater, Netherlands) (Day 2012) or (3 mg/kg SID p.o., Nycomed® 25 mg-pills, Takeda Austria GmbH, Vienna, Austria) or Methylprednisolone acetate 40 mg/ml plus Lidocaine hydrochloride 10 mg/ml (20 mg s.c., Depo-Medrol® with Lidocaine injection, Pfizer Limited, Sandwich, Kent, UK) (Day, 2012)

case no	abdominal sonography	FeLV, FIV	urine analysis	feces examination	Vitamin B12
1	yes	uk	no	no	uk
2	obstruction of the gastric exit, enlargened gastric walls, later liver infiltration, infiltration in caudal abdomen	no	no	no	no
3	thickening and loss of layering of the gastric wall, lymphadoenopathy of the gastric lnn.	negative	specific weight decreased	no	no
4	abdominal mass: inhomogenoeous with central hyperechogenic tissue--> ln.?	uk	uk	uk	uk
5	uk	uk	uk	uk	uk
6	uk	uk	uk	uk	uk
7	splenomegaly, abdominal lymphadenopathy	uk	uk	uk	uk
8	enlargened gastric walls	uk	uk	uk	uk
9	small intestinal mass, mesenteric lymphadenomegaly/-pathy, low-grade urine particles	negative	specific weight decreased	no	increased
10	gastric wall infiltration and mild lymphadenomegaly	negative	unremarkable	no	unremarkable
11	hepatomegaly, ascites, infiltration of the right lung	uk	uk	uk	uk
12	high grade small intestine infiltrations, high grade mesenteric lymphadenomegaly, nephropathy	negative	specific weight decreased	flotation and giardia snap-test negative	increased
13	mild ascites, enlarged abdominal ln., inhomogeneous spleen	uk	uk	uk	uk
14	abdominal mass, infiltration of the intestinal wall	no	yes	no	increased
15	neoplasia in small intestinal wall, renal infiltrations	negative	yes	no	decreased
16	uk	uk	uk	uk	uk
17	pancreatitis, hepatopathy, enlarged wall of the gallbladder	no	specific weight decreased	no	increased
18	infiltration of the gastric wall, questionnable infiltration of the mesenterium and the omentum	negative	specific weight increased	no	yes: increased
19	uk	uk	uk	uk	uk
20	uk	uk	uk	uk	uk
21	uk	uk	uk	uk	uk
22	uk	uk	uk	uk	uk
23	gastric wall infiltration, small intestinal wall infiltration, nephrosis	negative	unremarkable	no	decreased
24	uk	uk	uk	uk	uk

case no	CBC plus coagulation status	thoracic X-ray	pulmo-rhinoscopy	CT	MRI
1	yes	yes	yes	yes	no
2	yes	uk	no	no	no
3	yes	yes	no	no	yes
4	uk	uk	uk	uk	uk
5	yes	uk	no	uk	uk
6	uk	uk	uk	uk	uk
7	yes	yes	no	no	no
8	uk	uk	uk	uk	uk
9	yes	partial lungatelectasis or lung infiltration,	no	no	no
10	yes	questionable cardiomegaly, spondylosis deformans	no	no	no
11	yes	neoplasia, atelectasis: consolidation of the right lung, obstruction of the upper airways: pharyngeal ballooning	no	no	no
12	yes	sternal lymphadenomegaly and questionable abdominal mass	no	no	no
13	uk	uk	uk	uk	uk
14	yes	questionable metastasis	no	no	no
15	yes	cardiomegaly	no	no	no
16	uk	uk	uk	uk	uk
17	yes	yes	no	no	no
18	yes	unremarkable	no	no	no
19	uk	uk	uk	uk	uk
20	uk	uk	uk	uk	uk
21	uk	uk	uk	uk	uk
22	uk	uk	uk	uk	uk
23	yes	yes	no	no	no
24	uk	uk	uk	uk	uk

case no	localization	source material	IHC	FCM	cytology	histopathology/ biopsy
1	mass of the soft palate	4x OS FNA	no	no	Lymphoma	no
2	gastric wall	4x OS FNA	no	no	Lymphoma	no
3	stomach	5 x 10^6 cells (scs)	BCL	BCL	Lymphoma	DLBCL
4	abdominal mass	4x OS FNA	no	no	Lymphoma	no
5	submandibular, praescapular, popliteal lnn.	1x OS FNA	no	no	Lymphoma	no
6	mass in front of shoulder joint	3x OS FNA	no	no	Lymphoma	no
7	mandibular ln.	2x OS FNA	no	no	Lymphoma	no
8	stomach	3x OS FNA	no	no	Lymphoma	no
9	solid material ileocaecal trap	OS Paraffine	BCL	BCL	Lymphoma	DLBCL
10	stomach and gastric lnn.	5 x 10^6 cells (scs)	BCL	BCL	Lymphoma	anaplastic DLBCL intermediate grade*
11	popliteal and mandibular lnn.	5x 10^6 cells (scs)	regular distribution	reactive	reactive hyperplasia	reactive
12	intestine wall/abdominal ln.	5 x 10^6 cells (scs)	BCL	BCL	Lymphoma	anaplastic DLBCL high grade*
13	abdominal ln.	5x OS FNA	no	no	Lymphoma	no
14	ileocaecal area/mass on the outside of the intestine	5 x 10^6 cells (scs)	no	BCL	Lymphoma	transmural intestinal BCL
15	intestine and mass on the outside of intestine	5 x 10^6 cells (scs)	BCL	no	Lymphoma	DLBCL in jejunum, liver and kidney
16	right mandibular mass	5x OS	no	no	Lymphoma	no
17	mandibular ln.	5 x 10^6 cells (scs)	regular distribution	reactive	reactive hyperplasia	reactive
18	stomach	5 x 10^6 cells (scs)	BCL	BCL	Lymphoma	high-grade* DLBCL in stomach and mesenterium with suspected focal infiltration of the ln
19	abdominal mass	1x OS FNA	no	no	Lymphoma	no
20	intestine	3x OS FNA	no	no	Lymphoma	no
21	mandibular ln.	5 x 10^6 cells (scs)	regular distribution	reactive	reactive hyperplasia	reactive
22	popliteal ln.	5 x 10^6 cells (scs)	regular distribution	reactive	reactive hyperplasia	reactive
23	stomach/jejunum	5 x 10^6 cells (scs)	BCL	BCL	Lymphoma	DLBCL intermediate grade *
24	mass or ln.	2x FNA	no	no	Lymphoma	no

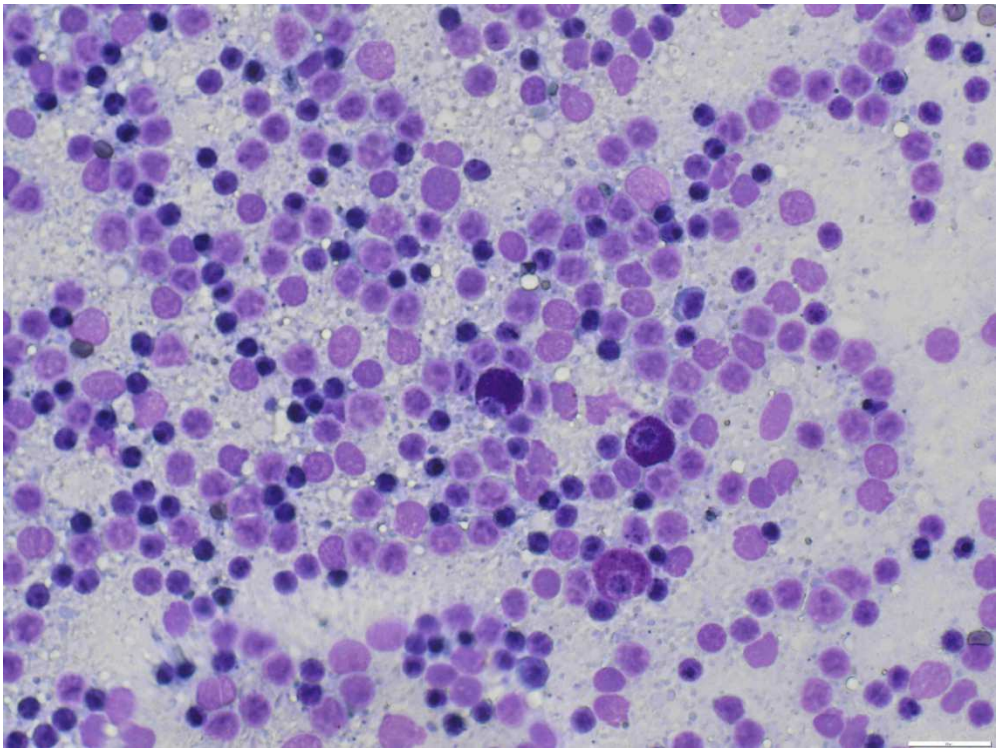
case no	symptomatic	chemotherapy	radiation
1	IFT, antibiotics, antiemetics, gastric protection	COP plus Cytarabin (800 mg/m^2 single injection, Alexan® Cytosar-500 mg/ml, Cikarang Bekasi, Indonesia)	palliative because of worsening
2	gastric protection	COP plus Doxorubicin (1 mg/kg i.v., Adriblastin 50 mg solution, Pharmacia GmbH, Berlin, Germany) plus L-Asparaginase (5000 E/m^2/day s.c., Asparaginase 5000 E Medac®, Medac, Wedel, Germany)	no
3	IFT, antiemetics, anticonvulsives, antibiotics, gastric protection, antibiotics, analgetics	COP	radiation because of renal and nasal involvment
4	uk	uk	uk
5	uk	uk	uk
6	uk	uk	uk
7	antibiotics, antiemetics, vitamin B12 substitution, gastric protection, diuretics	COP plus Cytarabin (800 mg/m^2 single injection, Alexan® Cytosar-500 mg/ml, Cikarang Bekasi, Indonesia)	no
8	uk	uk	uk
9	IFT, gastric protection, analgetics, antiemetics, antibiotics, diuretics, appetizers	COP	planned radiation
10	IFT, gastric protection, analgetics, antiemetics, antibiotics, diuretics, appetizers, thyreostatic, vitamin B12 substitution, blood transfusion	COP	uk
11	IFT, antiemetics, gastric protection	no	no
12	IFT, antibiotics, analgetics, antiemetics, appetizers, gastric protection, phosphor binder, vitamin B12 substitution	COP plus L-Asparaginase (5000 E/m^2/day s.c., Asparaginase 5000 E Medac®, Medac, Wedel, Germany)	no
13	uk	uk	uk
14	IFT, analgetics, antiemetics, antibiotics, gastric protection, laxatives, appetizers, blood transfusion, diuretics, Angiotensin converting enzyme inhibitors, synthetic Erythropoietin	COP	no
15	IFT, antiemetics, gastric protection	no	no
16	uk	uk	uk
17	IFT, Angiotensin converting enzyme inhibitors, antibiotics, gastric protection, antiemetics, liver regeneration promoters	no	no
18	IFT, analgetics, antibiotics, gastric protection, antiemetics, synthetic catecholamine, blood plasma substitute	no	no
19	uk	uk	uk
20	uk	uk	uk
21	no	no	no
22	no	no	no
23	IFT, antibiotics, analgetics, vitamin B12 substitution, appetizers, lactic acid bacterias, Psyllium husks	COP	no
24	uk	uk	uk

case no	surgical therapy	survival time
1	no	5 months
2	no	3 months
3	partial gastrectomy , oesophagogastric tube	3 months
4	uk	uk
5	uk	uk
6	uk	uk
7	no	1,5 months
8	uk	uk
9	enterectomy plus oesopagogastric tube	2 months
10	partial gastrectomy: macroscopical mass completely removed with all enlarged lnn. plus oesopaghogastric tube	2 years and 5 months
11	no	2 days
12	enterectomy and colectomy plus oesophagaogstric tube, mass of 300 g in the ileocaecal region with enlargened ln. was removed	1 year
13	uk	uk
14	enterectomy (ileocaecal region and a mass on the outside of the colon were removed, but a mass in the rectum could not be removed: pelvis must be opened during 2nd surgery: osteotomy, but could still not be removed) oesophagogastric tube	10 months
15	no	1 day
16	uk	uk
17	no	3 day
18	yes: gastrectomy: removal of a 7 cm mass in the stomach to 1 cm in front of the pylorus and 3 cm in front of the cardia and removal of the mesenterium, partial splenectomy, oesophagogastric tube	6 days
19	uk	uk
20	uk	uk
21	no	uk
22	no	uk
23	enterectomy (part resection of the stomach and of one enlarged gastric ln.)	3 months
24	uk	uk

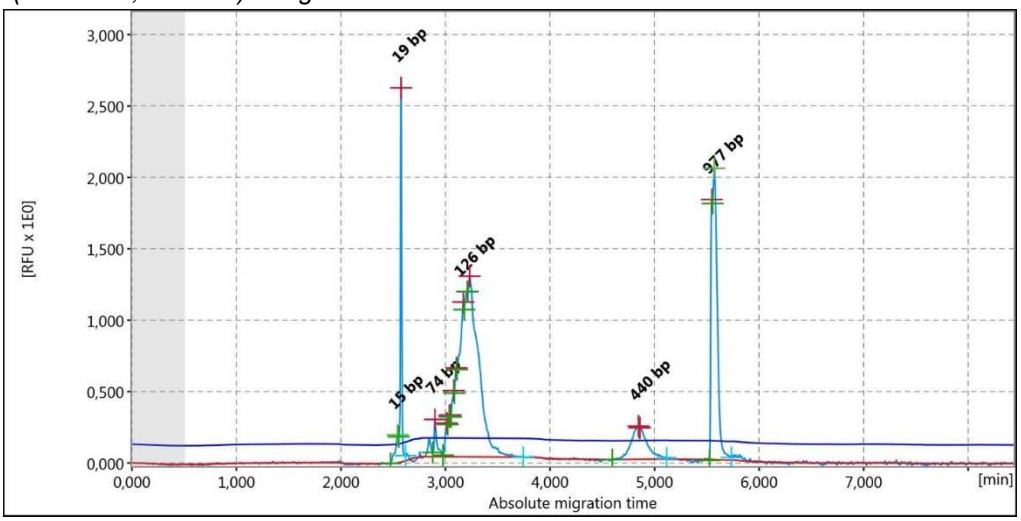
case no	cause of death	sectio
1	14.06.15 euthanasia because of acute worsenening, anorexia, neutropenia (recurrence of LSA or post Cytarabin (800 mg/m2 single injection, Alexan® Cytosar-500 mg/ml, Cikarang Bekasi, Indonesia))	yes: multicentric atypical lymphosarcoma, stade Vb with renal and hepatical involvment, acute renal insufficiency
2	21.06.15 euthanasia because of relapse	no
3	07.12.16 euthanasia due to breathing arrest	yes: DLBCL, mid lobe infiltration, aspiration, pneumonia, renal involvment, nasal lymphoma
4	uk	uk
5	uk	uk
6	uk	uk
7	28.10.2017 euthanasia because of ascites, weakness and anemia by the house vet	yes: nodal lympoma, peritoneal effusion, splenomegaly
8	uk	uk
9	20.04.18 euthanasia because of dyspnoa because of liquidothorax	yes: anaplastic DLBCL with manifestation in sternal and mesenteric lymphnodes, liver, pancreas and bone marrow
10	no	yes: hyperthyreosis, anaplastic LBCL intermediate grade
11	26.06.18 euthanasia because of poor prognosis and bad condition	no
12	uk	uk
13	uk	uk
14	12.09.19 euthanasia because of renal insufficiency and haemolytic anemia, IBD	yes: chronic renal insufficiency with renal hyperparathyreoidismus and consecutive mineralisation of the gastric mucosa and aorta, lymphoplasmacytic enteritis and concentric left ventricular hypertrophia and myelopathy
15	05.04.20 euthanasia because owners decided against therapy	yes: DLBCL with manifestation in jejunum, liver and kidney
16	uk	uk
17	21.05.2019 euthanasia because of bad condition (no urine, hypothermia)	no
18	15.07.2019 breathing arrest during second surgery because of septic peritonitis	no
19	uk	uk
20	uk	uk
21	allergic shock: died peracutely	uk
22	facial swelling, inflammation	uk
23	no	no
24	uk	uk

Supplementary Figure 2. Non-neoplastic cases

Case 11

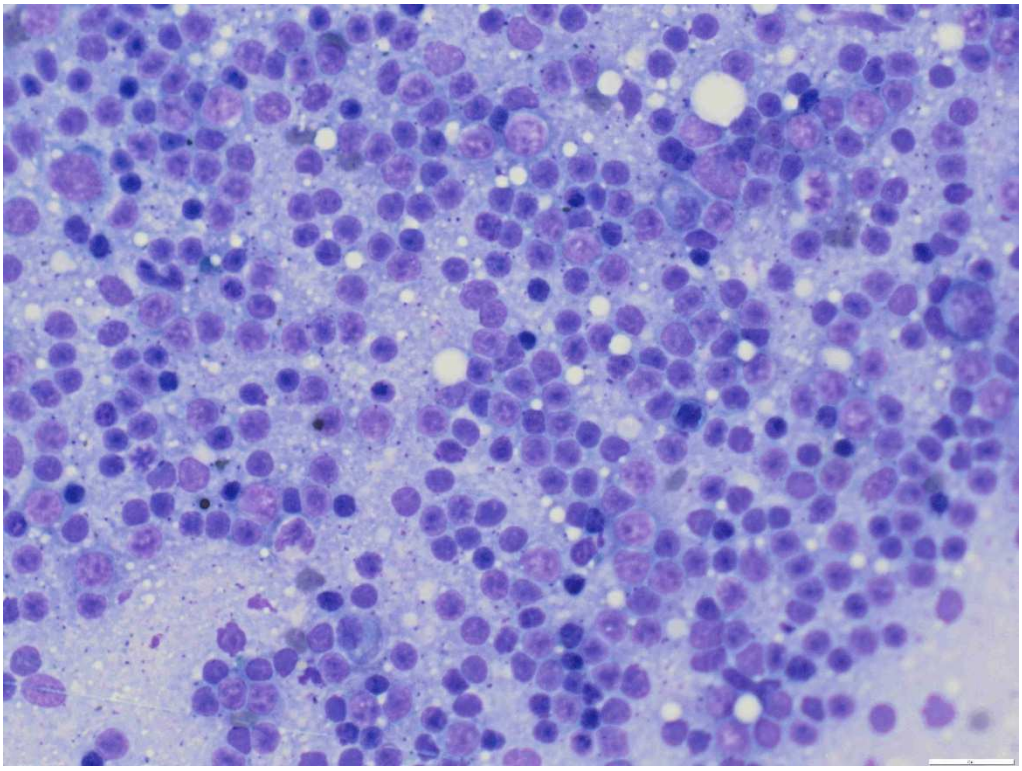


Case no. 11 (sample no. 11): Cytology specimen with a C2 classification. Localization: Popliteal and mandibular lymph node. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.

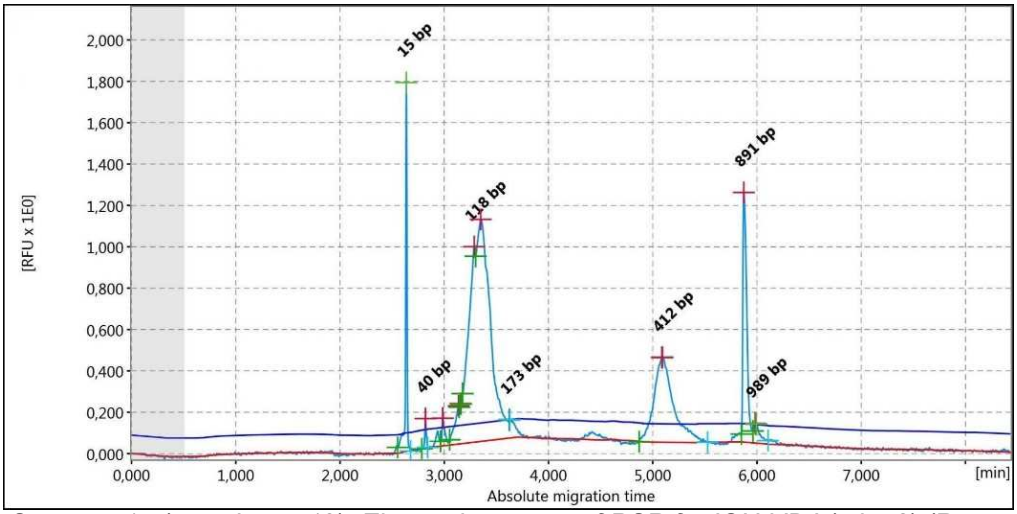


Case no. 11 (sample no. 11): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a polyclonal pattern around 126 bp.

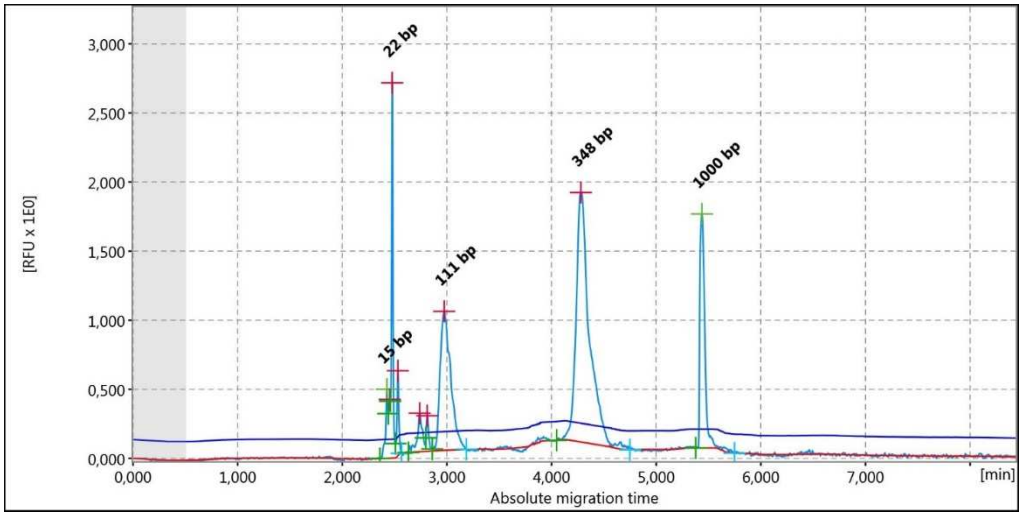
Case 17



Case no. 17 (sample no. 19): Cytology specimen with a C2 classification. Localization: Mandibular lymph node. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.

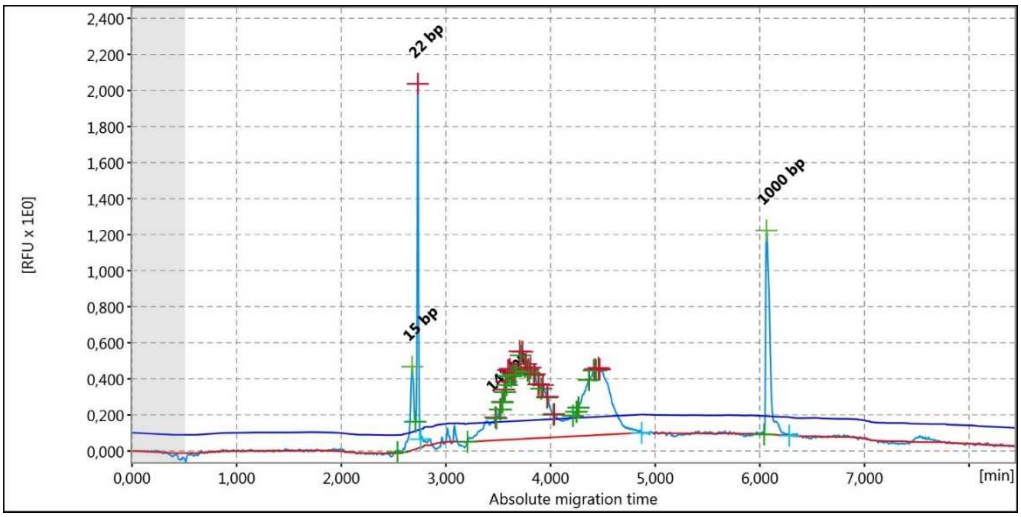


Case no. 17 (sample no. 19): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with polyclonal patterns at around 118 and 412 bp.

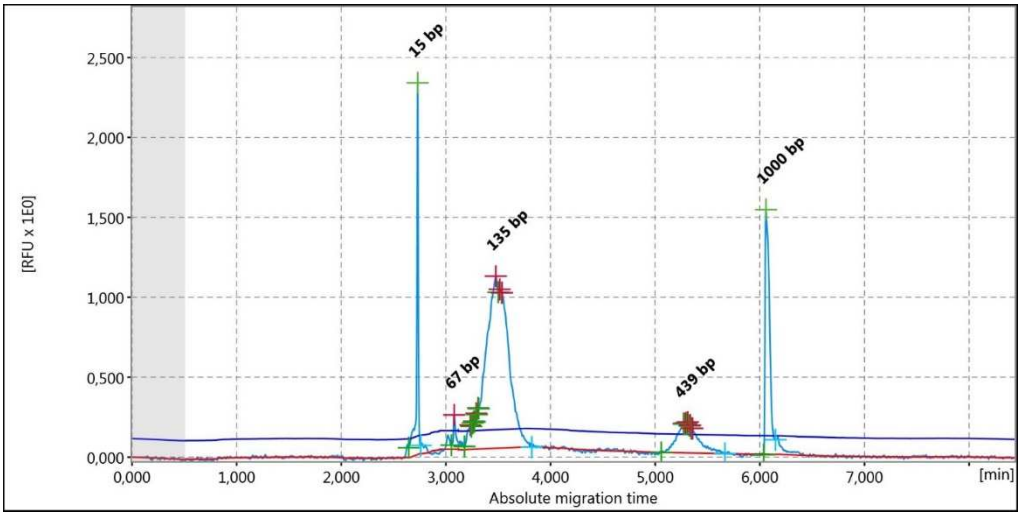


Case no. 17 (sample no. 19): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with polyclonal patterns at around 111 (IGH-DJ) and 348 (IGL) bp.

Case 21

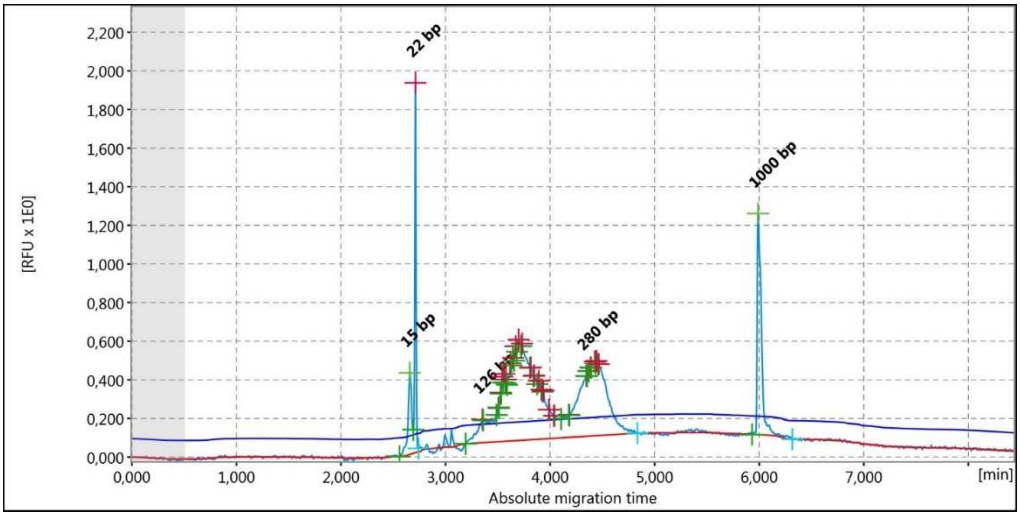


Case no. 21 (sample no. 23): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a polyclonal pattern around 141 and 295 bp

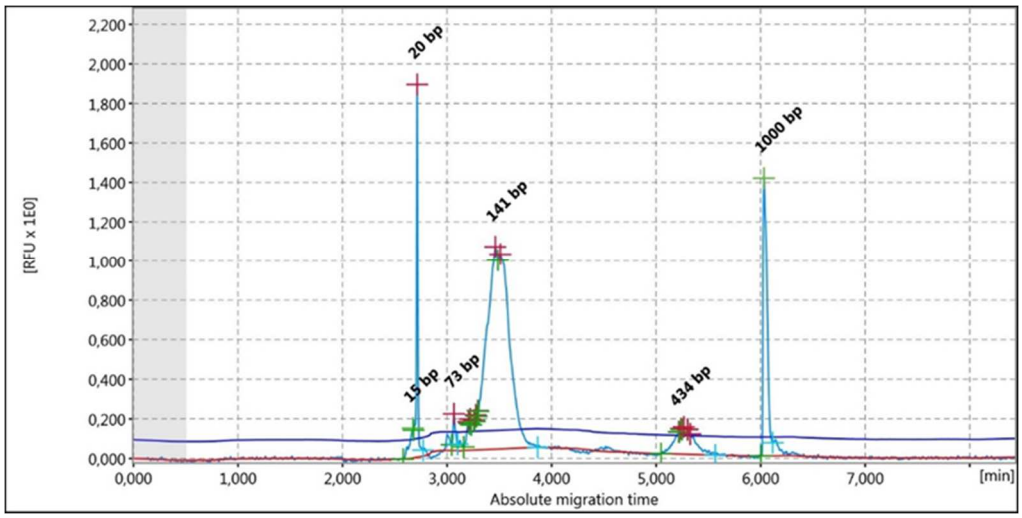


Case no. 21 (sample no. 23): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a polyclonal pattern around 135 bp.

Case 22



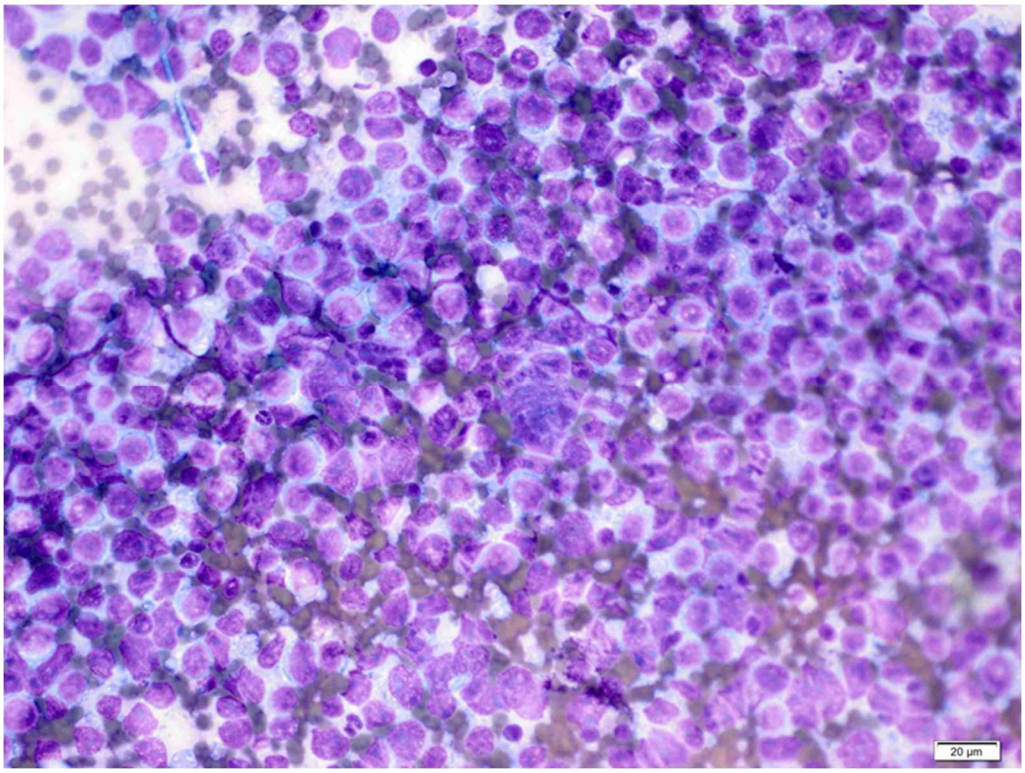
Case no. 22 (sample no. 24): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a polyclonal pattern around 126 bp and 280 bp.



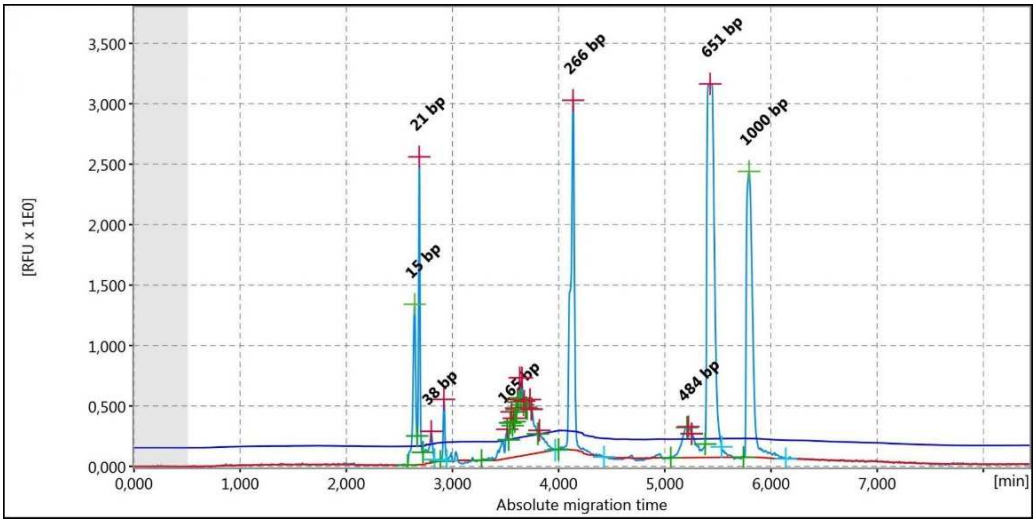
Case no. 22 (sample no. 24): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a polyclonal pattern around 141 bp.

Supplementary Figure 2. Neoplastic cases

Case 1

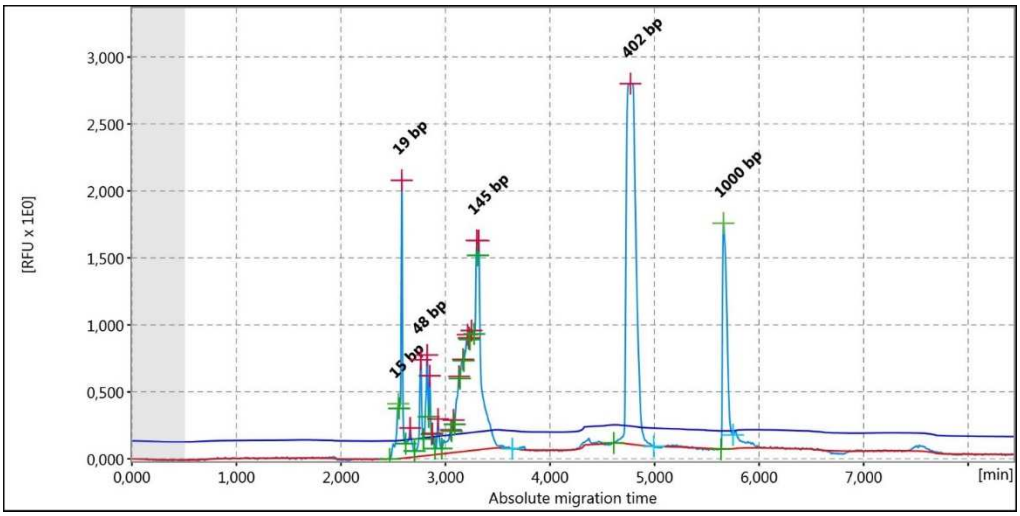


Case no. 1 (sample no. 1): Cytology specimen with a C5 classification. Localization: Soft palate. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.



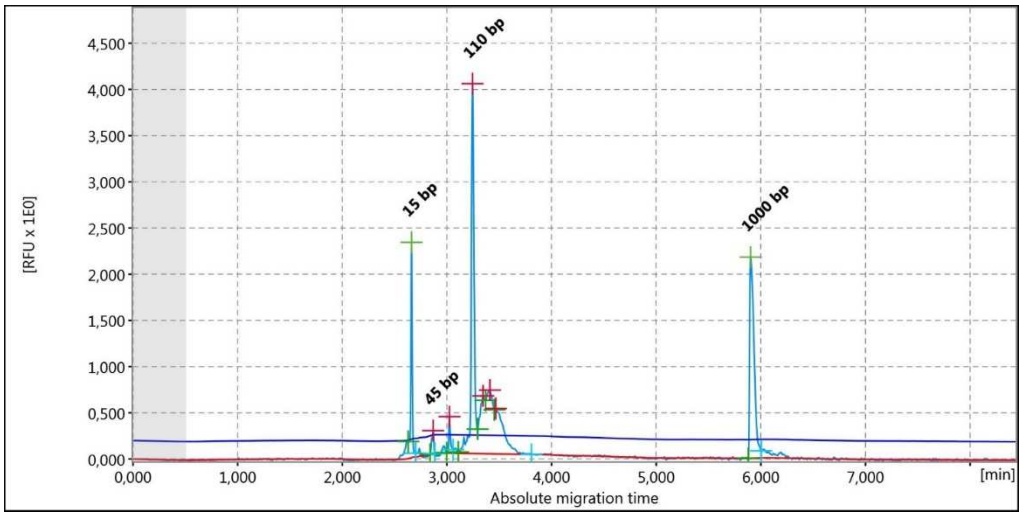
Case no. 1 (sample no. 1): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 266 bp.

Case 2

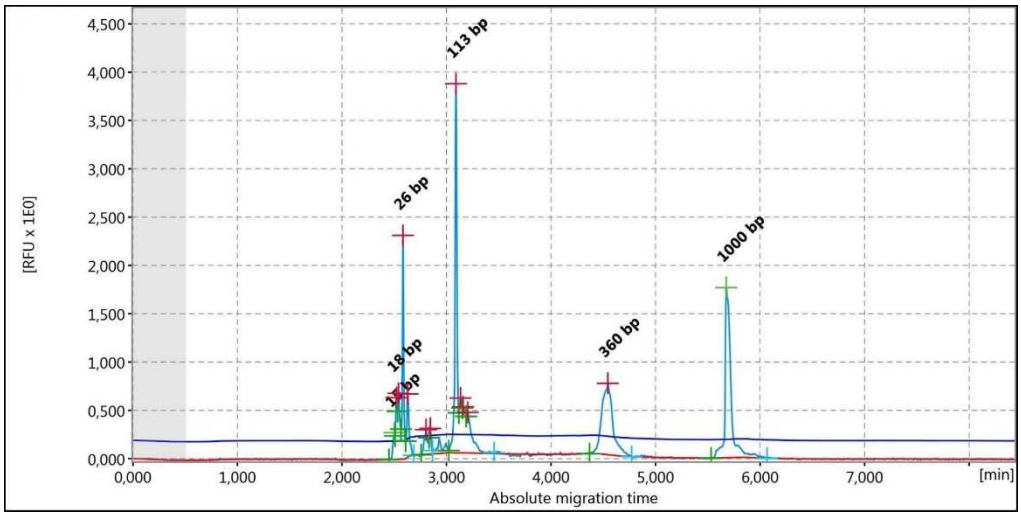


Case no. 2 (sample no. 2): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a polyclonal pattern around 145 bp showing a questionable monoclonal peak.

Case 3

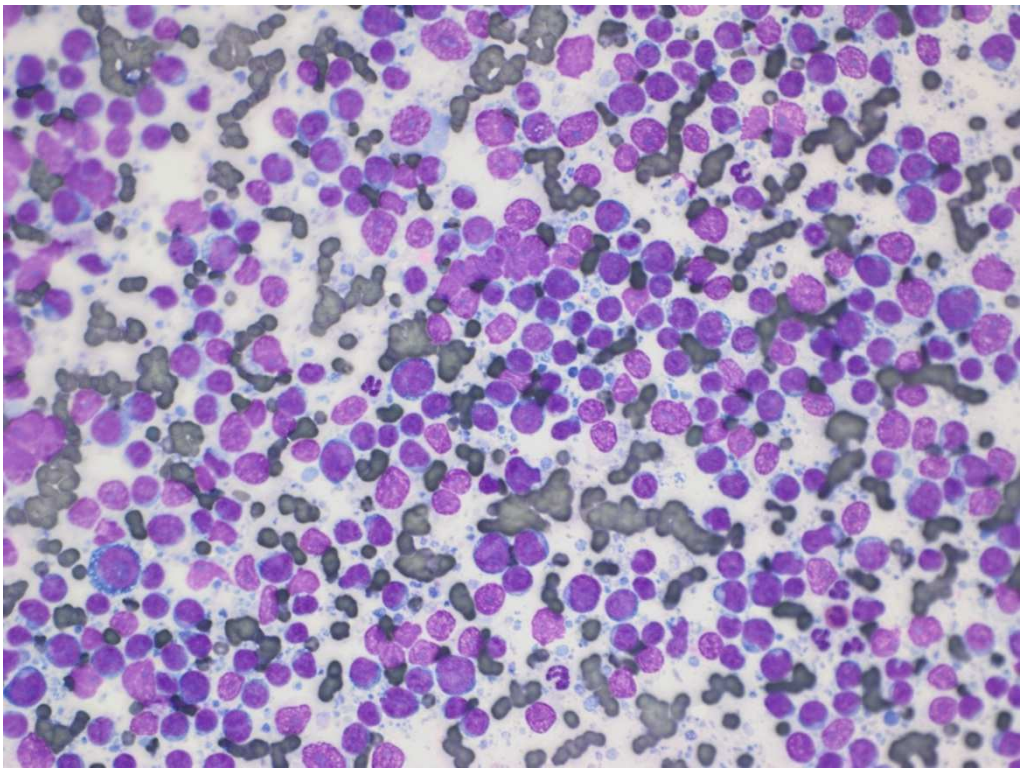


Case no. 3 (sample no. 3): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a monoclonal peak at 110 bp in a polyclonal background.

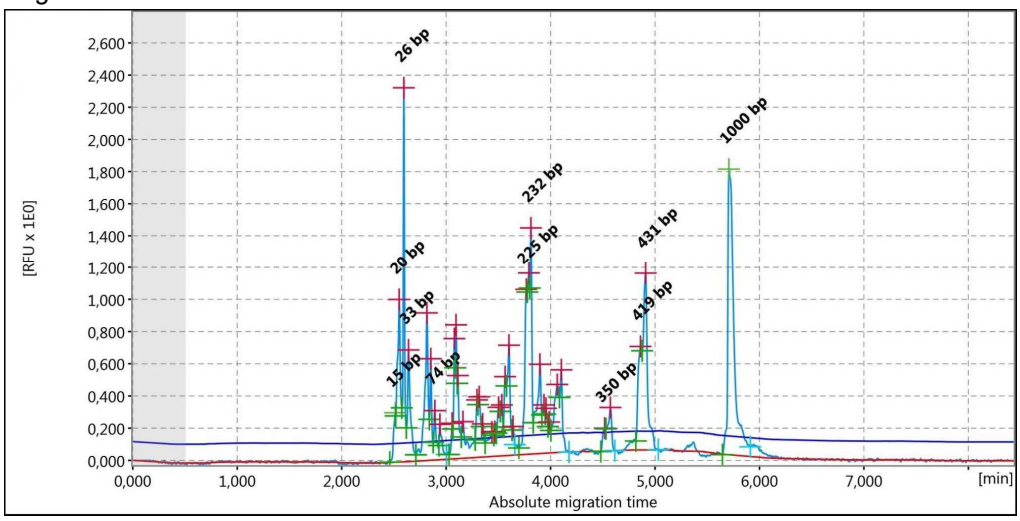


Case no. 3 (sample no. 3): Electropherogram of singleplex PCR for IGH-DJ (Rout et al., 2019) with a monoclonal peak at 113 bp (IGH-DJ) in a polyclonal background.

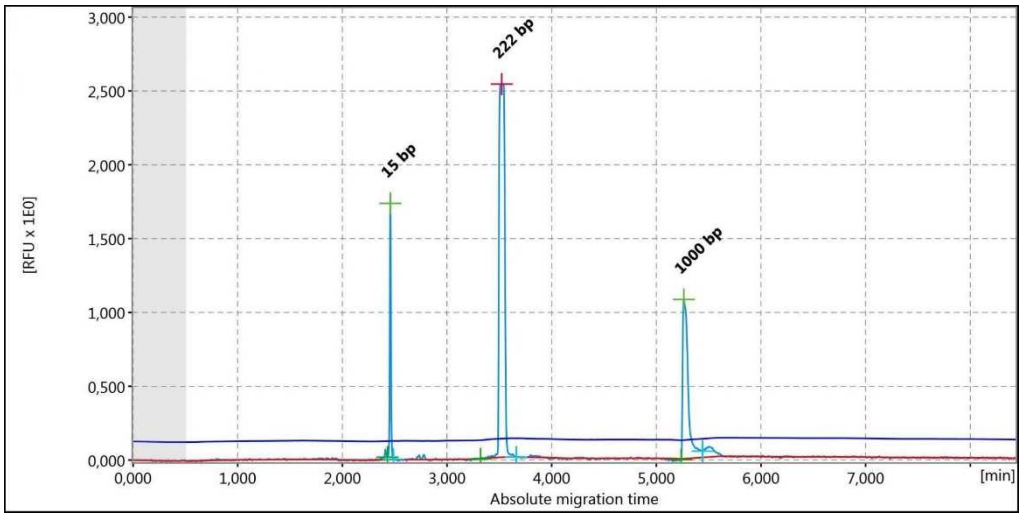
Case 4



Case no. 4 (sample no. 4): Cytology specimen with a C5 classification. Localization: Abdomen. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x

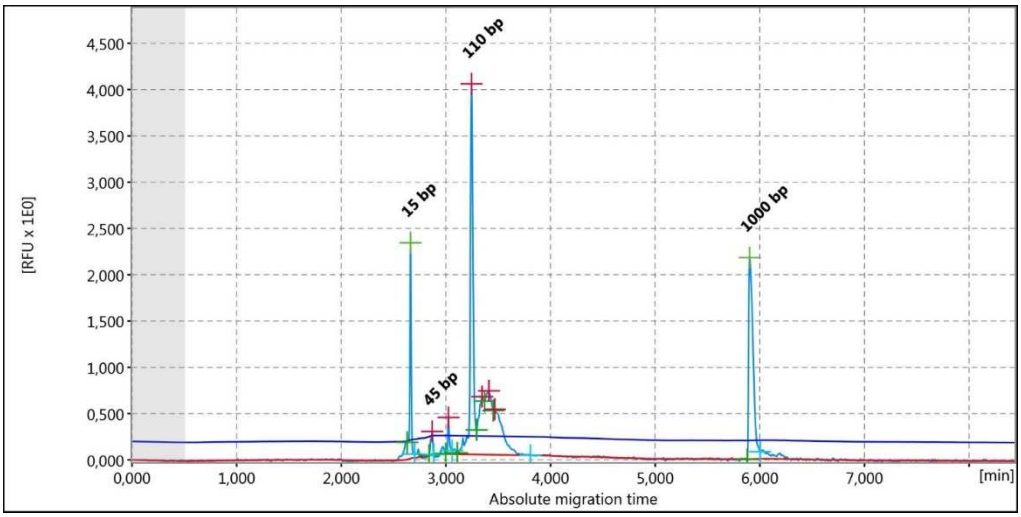


Case no. 4 (sample no. 4): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with a monoclonal peak at 232 bp (Kde) in a polyclonal backgroud.

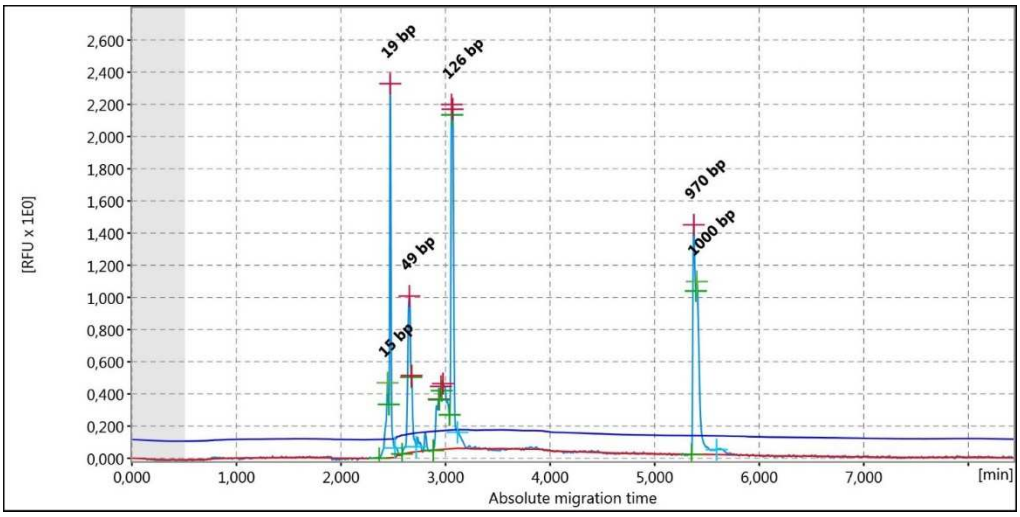


Case no. 4 (sample no. 4): Electropherogram of singleplex PCR for Kde (Rout et al., 2019) with a monoclonal peak at 222 bp (Kde).

Case 5

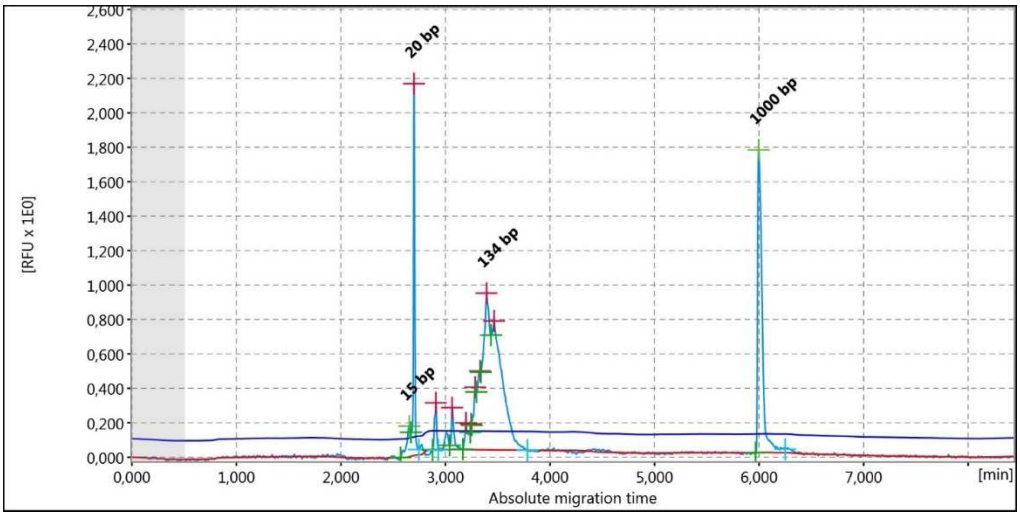


Case no. 5 (sample no. 5): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a monoclonal peak at 110 bp in a polyclonal background.



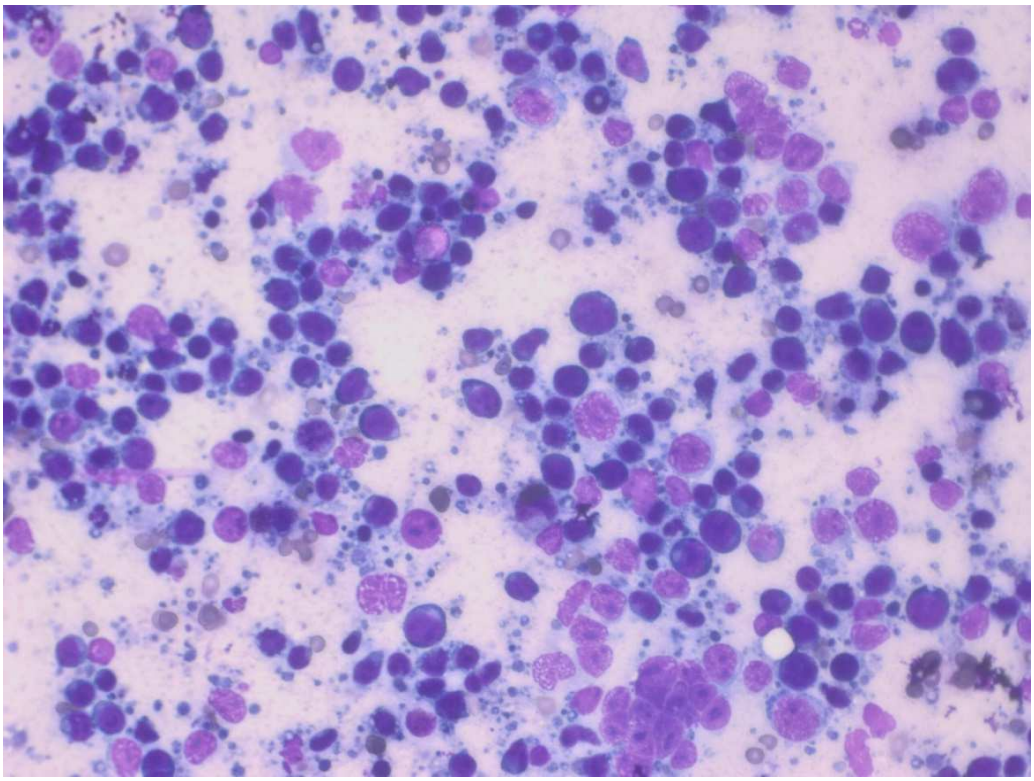
Case no. 5 (sample no. 5): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with a monoclonal peak at 126 bp (IGH-DJ) in a polyclonal background.

Case 6

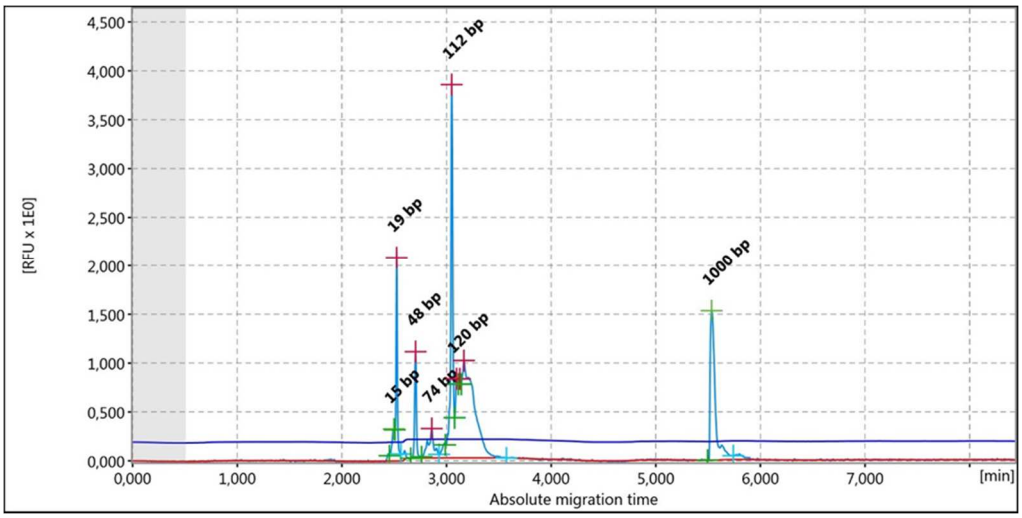


Case no. 6 (sample no. 6): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a polyclonal pattern around 134 bp.

Case 7

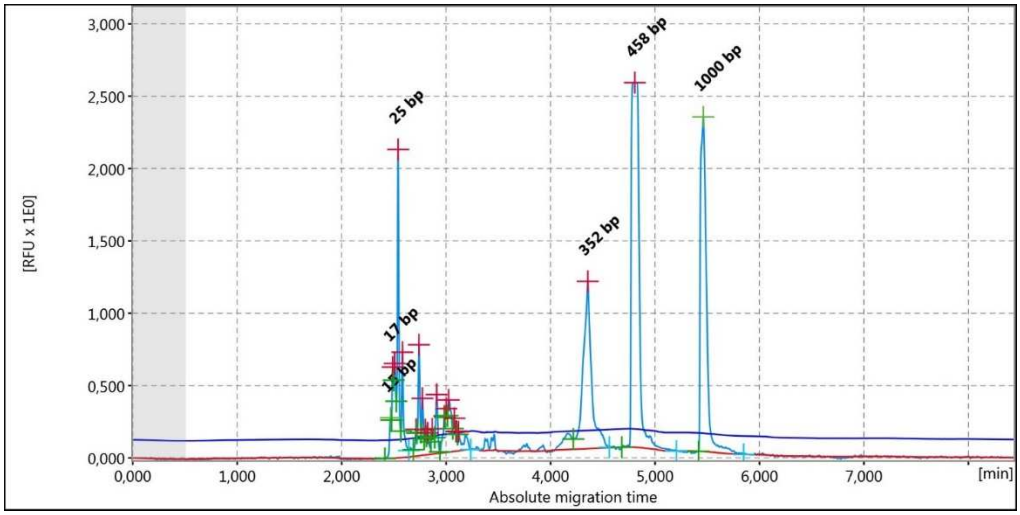


Case no. 7 (sample no. 7): Cytology specimen with a C5 classification. Localization: Mandibular lymph node. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.



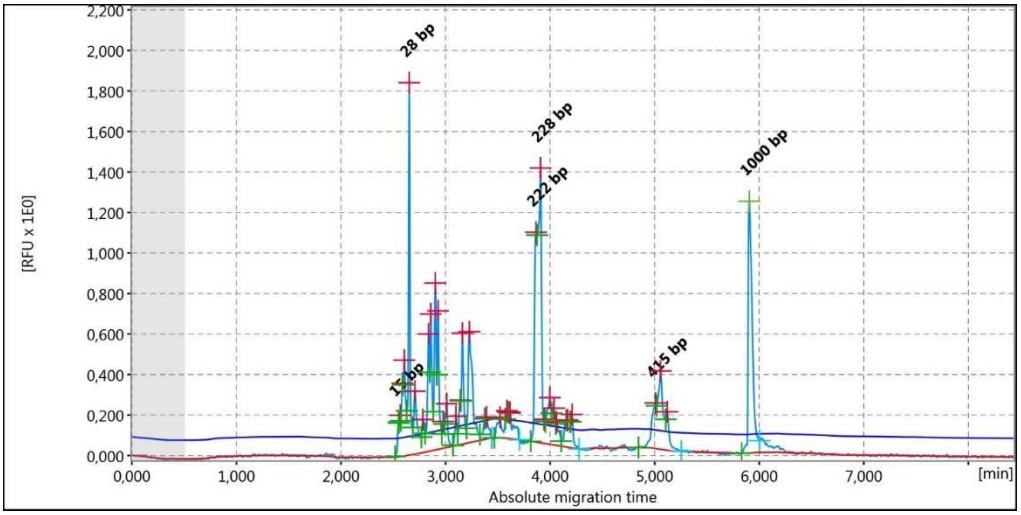
Case no. 7 (sample no. 7): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a monoclonal peak at 112 bp in a polyclonal background.

Case 8



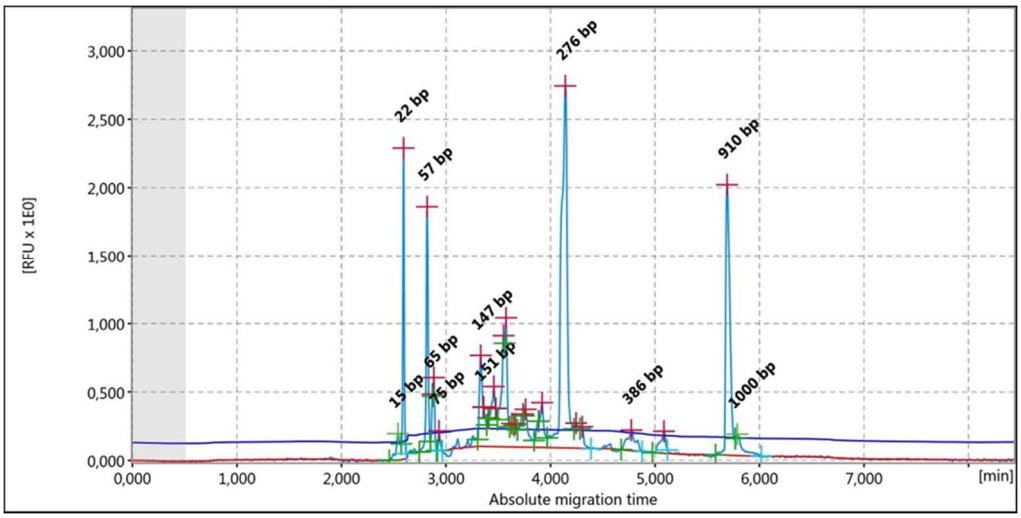
Case no. 8 (sample no. 8): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a polyclonal pattern around 100 bp.

Case 9



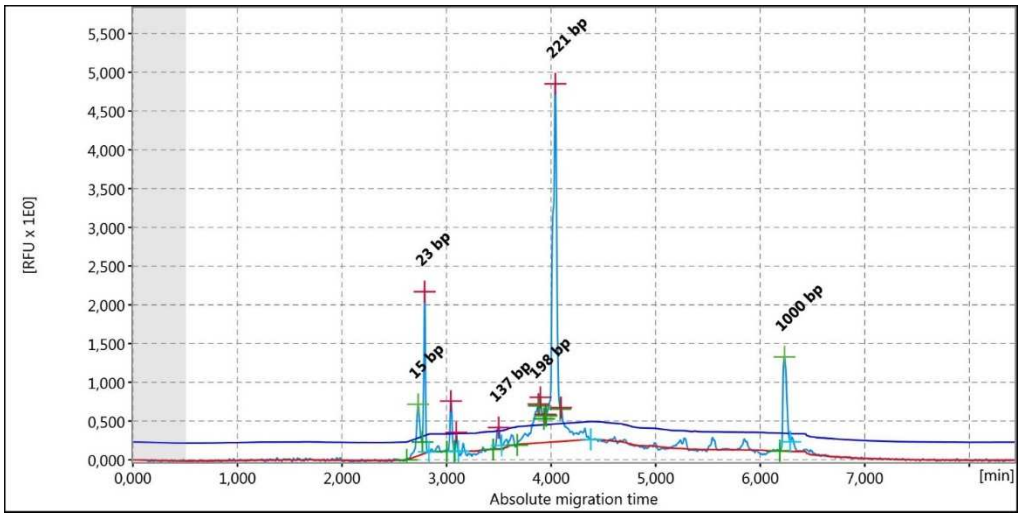
Case no. 9 (sample no. 9): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with a monoclonal peak at 228 bp (Kde).

Case 10

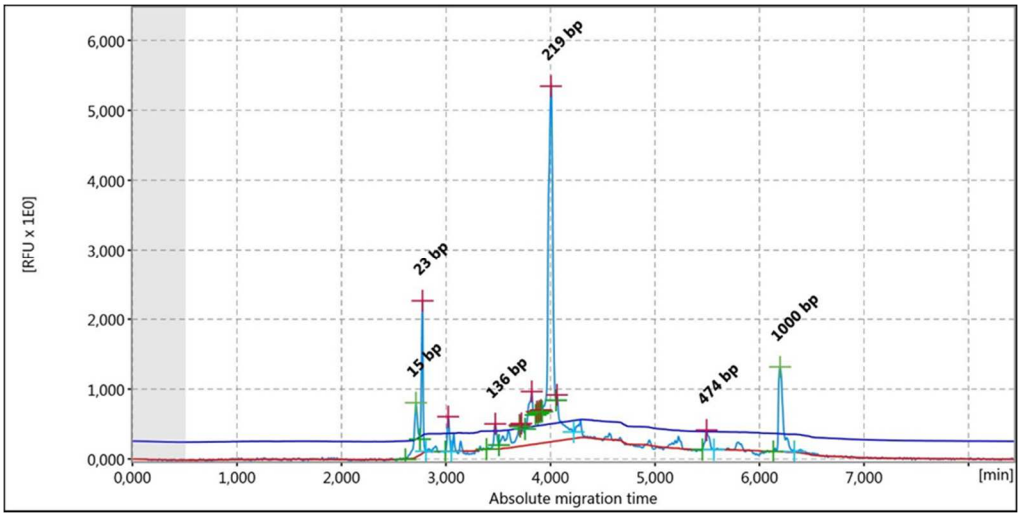


Case no. 10 (sample no. 10): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 276 bp.

Case 12

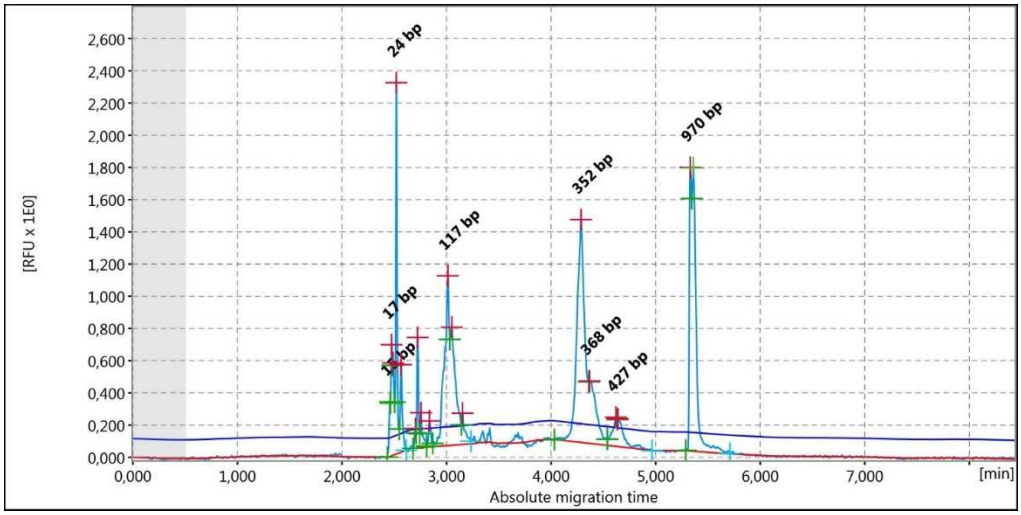


Case no. 12 (sample no. 12): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 221 bp.



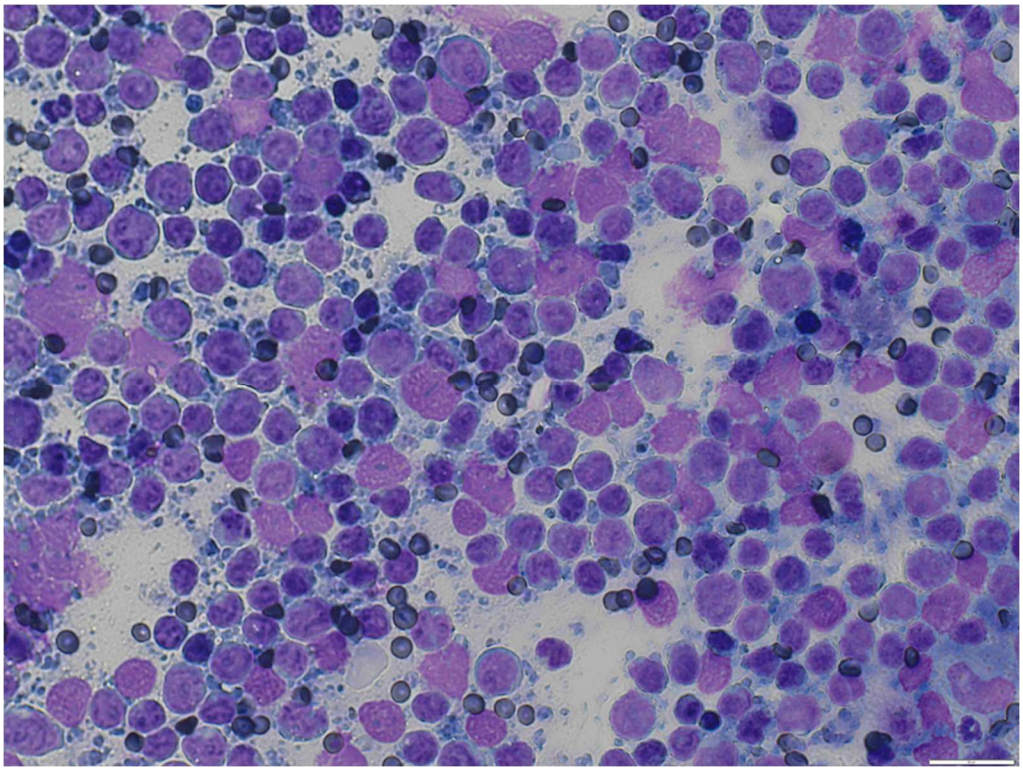
Case no. 12 (sample no. 13): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 219 bp.

Case 13

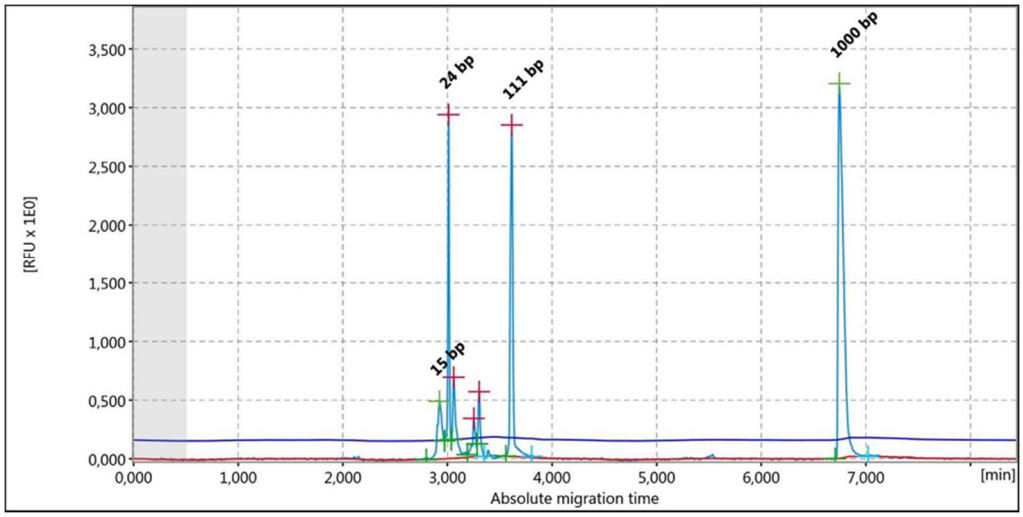


Case no. 13 (sample no. 14): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a polyclonal pattern around 117 and 352 bp.

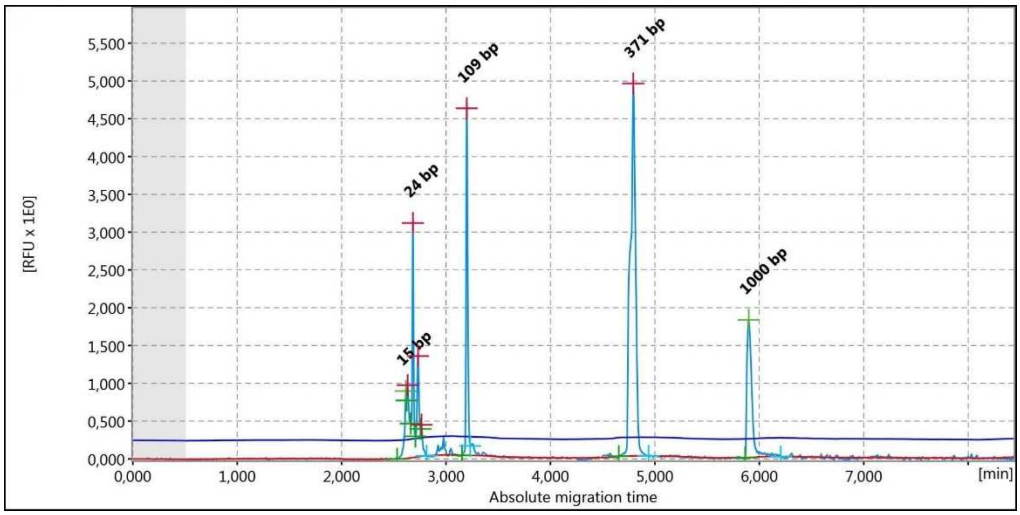
Case 14



Case no. 14 (sample no. 16): Cytology specimen with a C5 classification. Localization: Mass on the outside of the intestine. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.

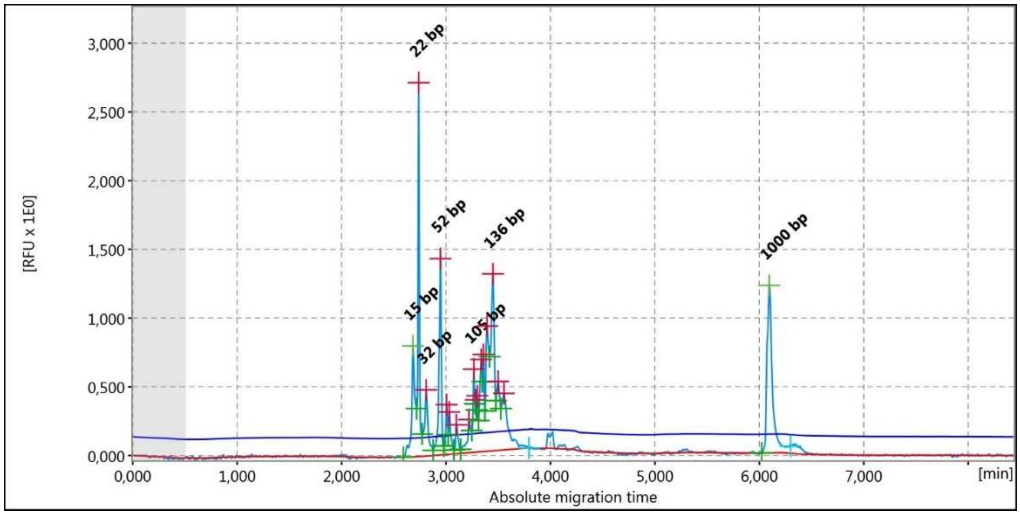


Case no. 14 (sample no. 15): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with a monoclonal peak at 111 bp (IGH-DJ).



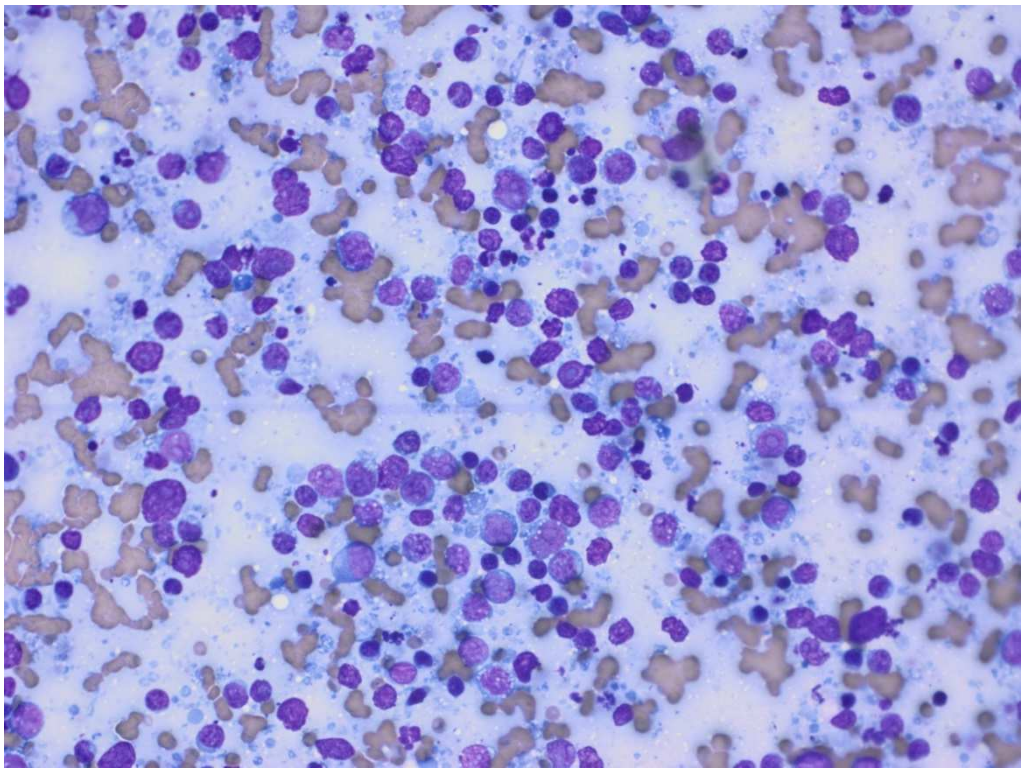
Case no. 14 (sample no. 16): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with monoclonal peaks at 109 bp (IGH-DJ) and 371 bp (IGL).

Case 15

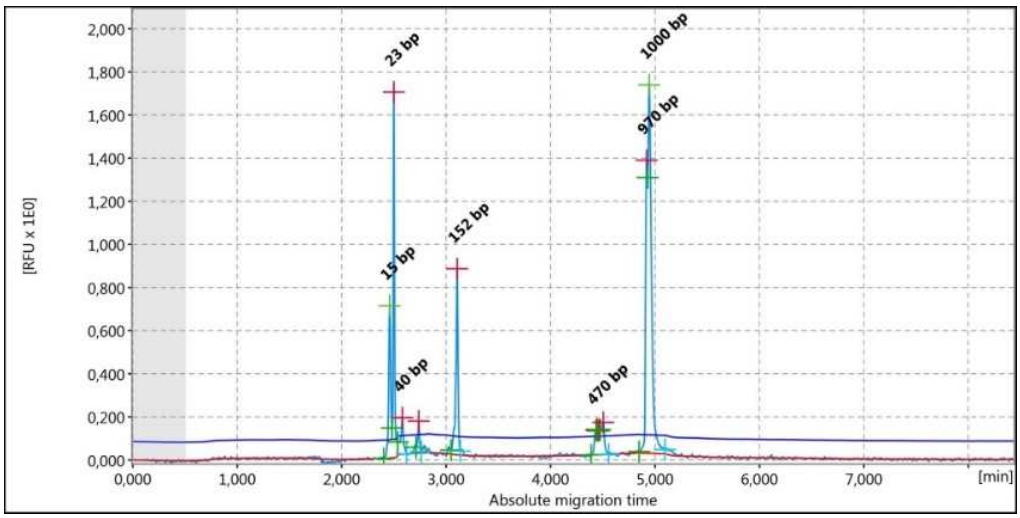


Case no. 15 (sample no. 17): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a polyclonal pattern around 136 bp showing a questionable monoclonal peak.

Case 16

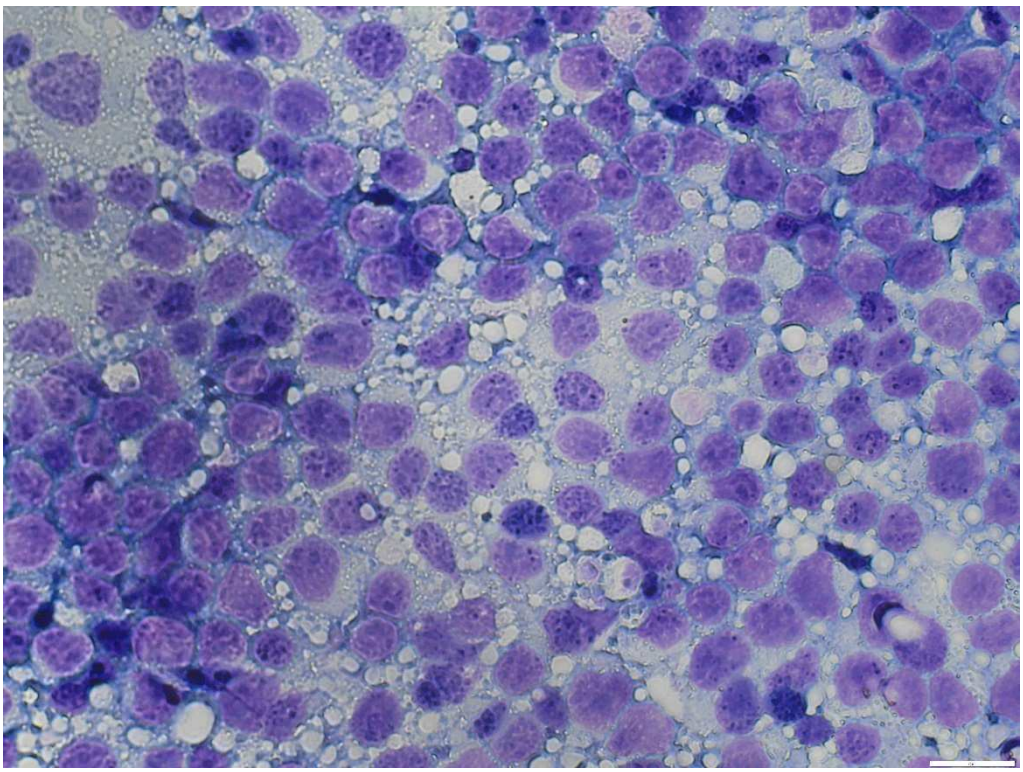


Case no. 16 (sample no. 18): Cytology specimen with a C5 classification. Localization: Mandibular mass. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.

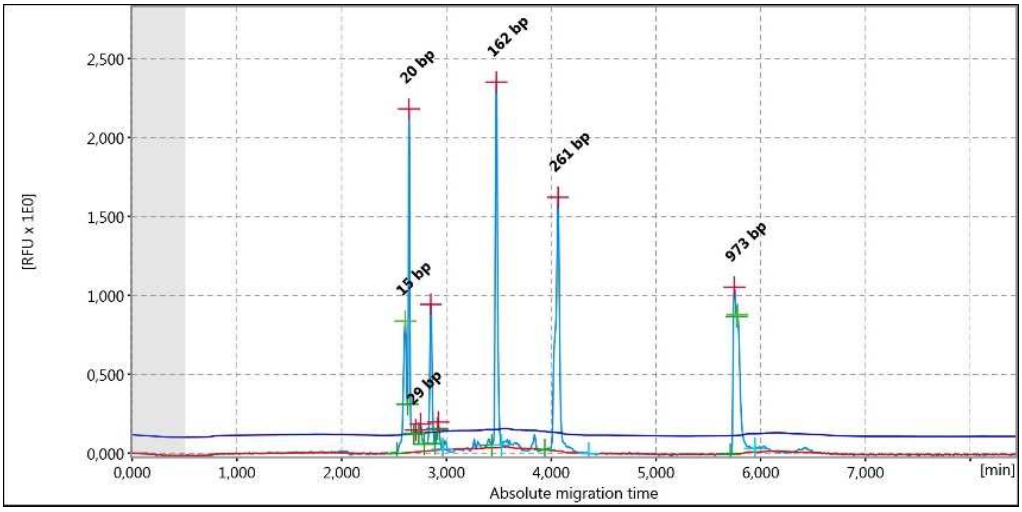


Case no. 16 (sample no. 18): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 152 bp.

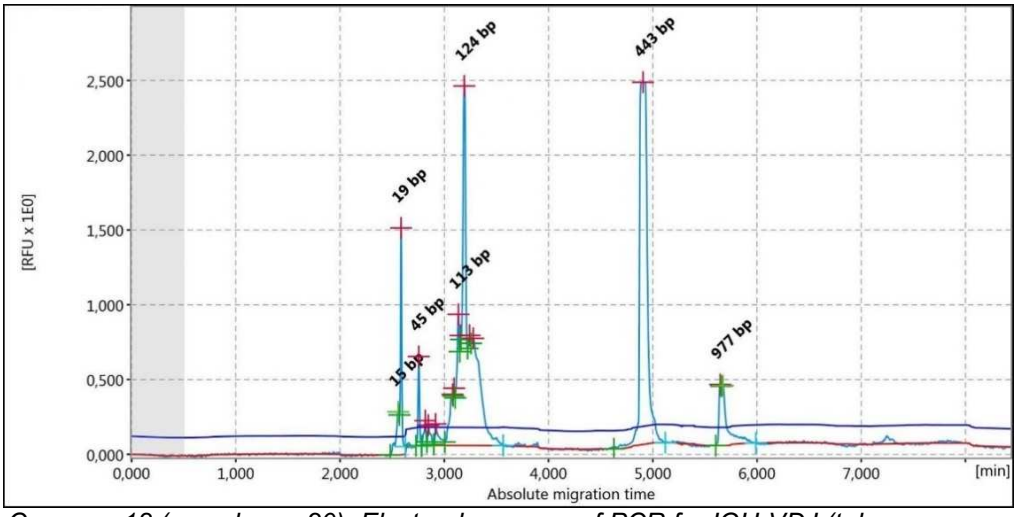
Case 18



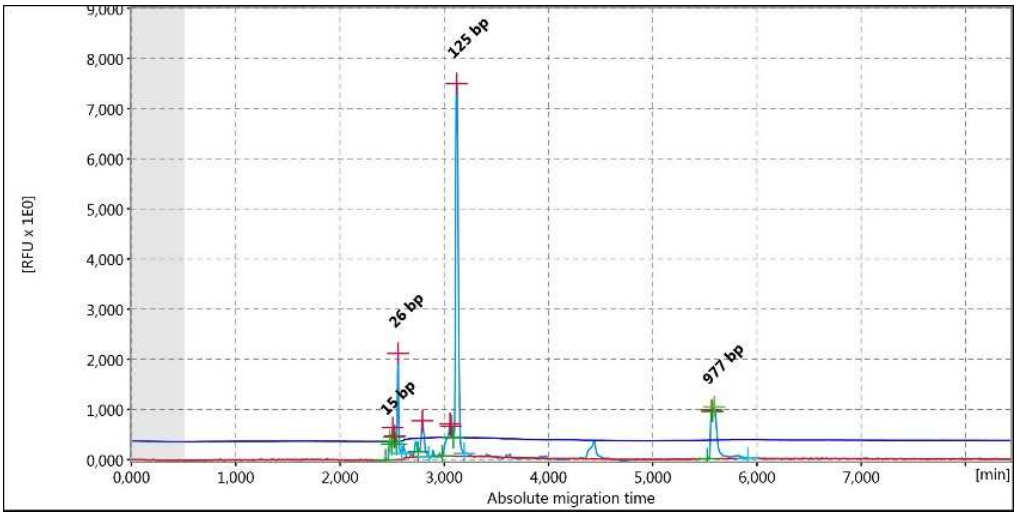
Case no. 18 (sample no. 20): Cytology specimen with a C5 classification. Localization: Stomach. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.



Case no. 18 (sample no. 20): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with biclonal peaks at 162 and 261 bp.

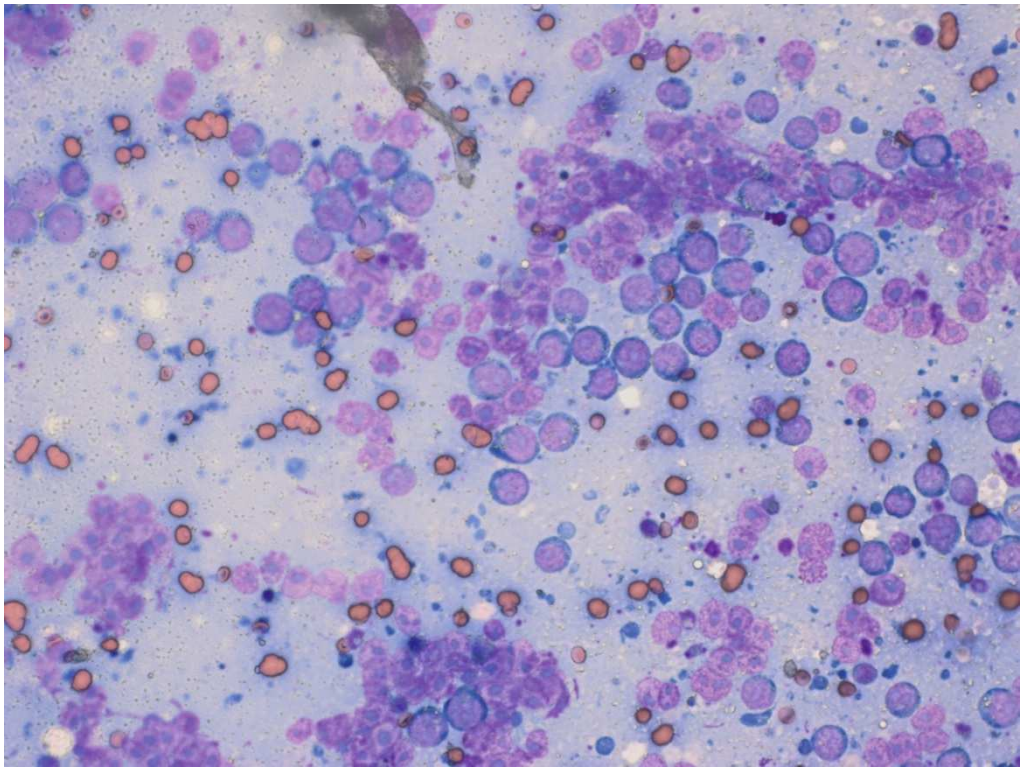


Case no. 18 (sample no. 20): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a monoclonal peak at 124 bp in a polyclonal background

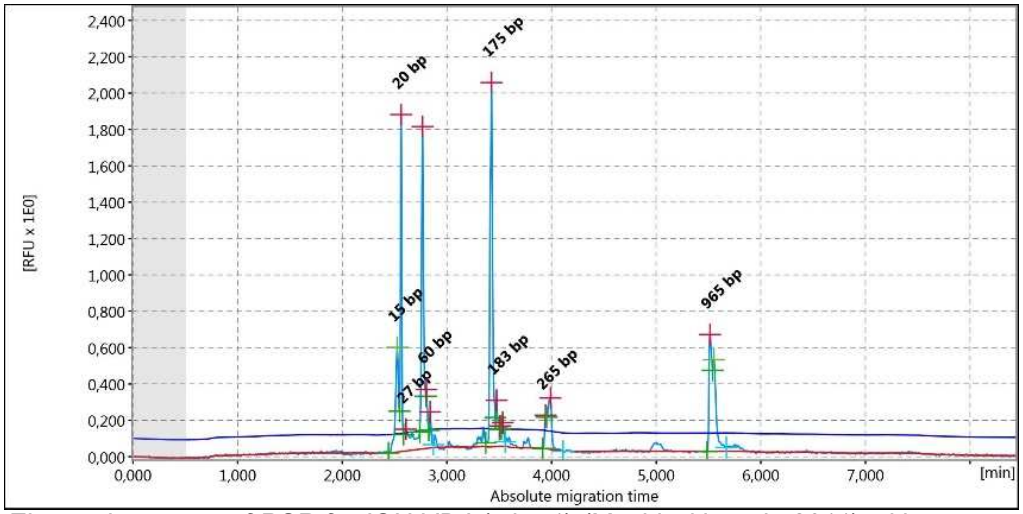


Case no. 18 (sample no. 20): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with a monoclonal peak at 125 bp (IGH-DJ).

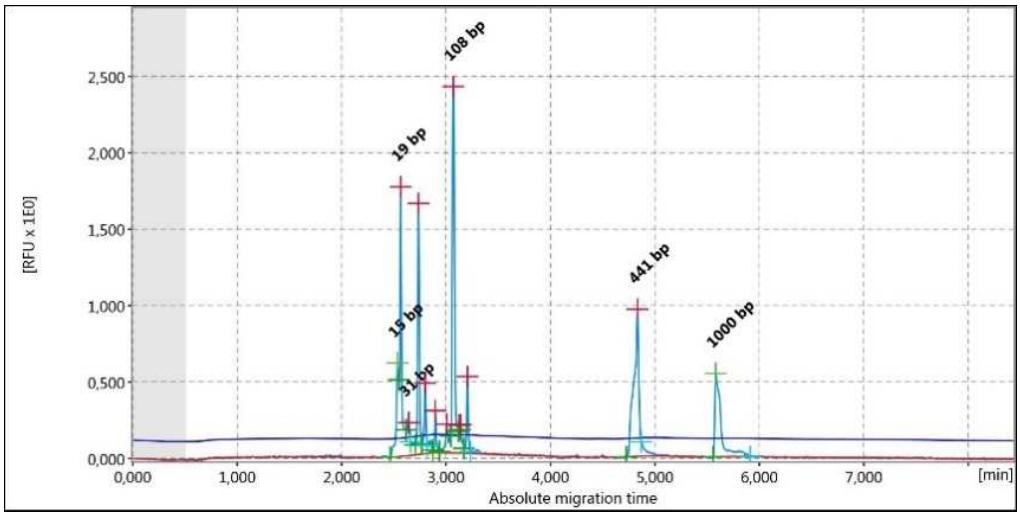
Case 19



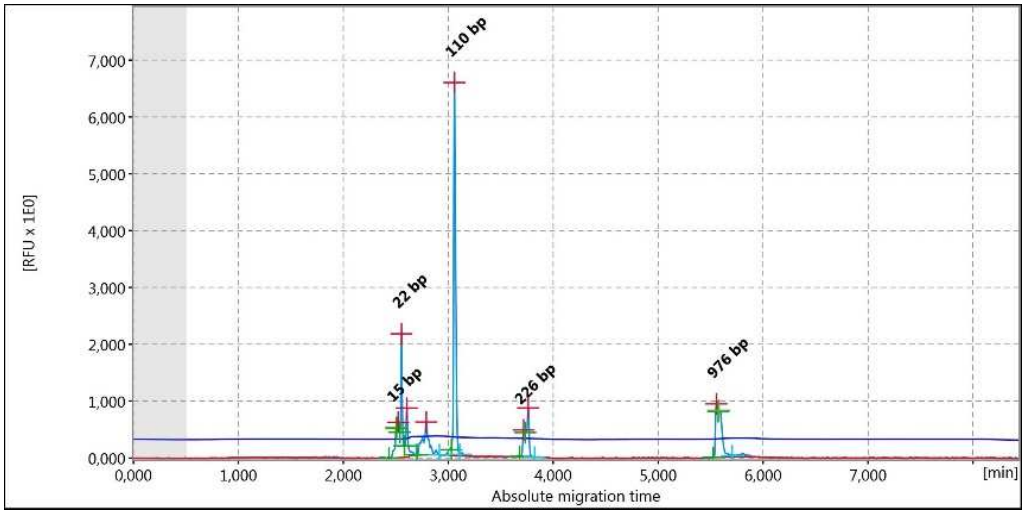
Case no. 19 (sample no. 21): Cytology specimen with a C5 classification. Localization: Abdominal mass. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100xCase no. 19 (sample no. 21):



Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 175 bp.

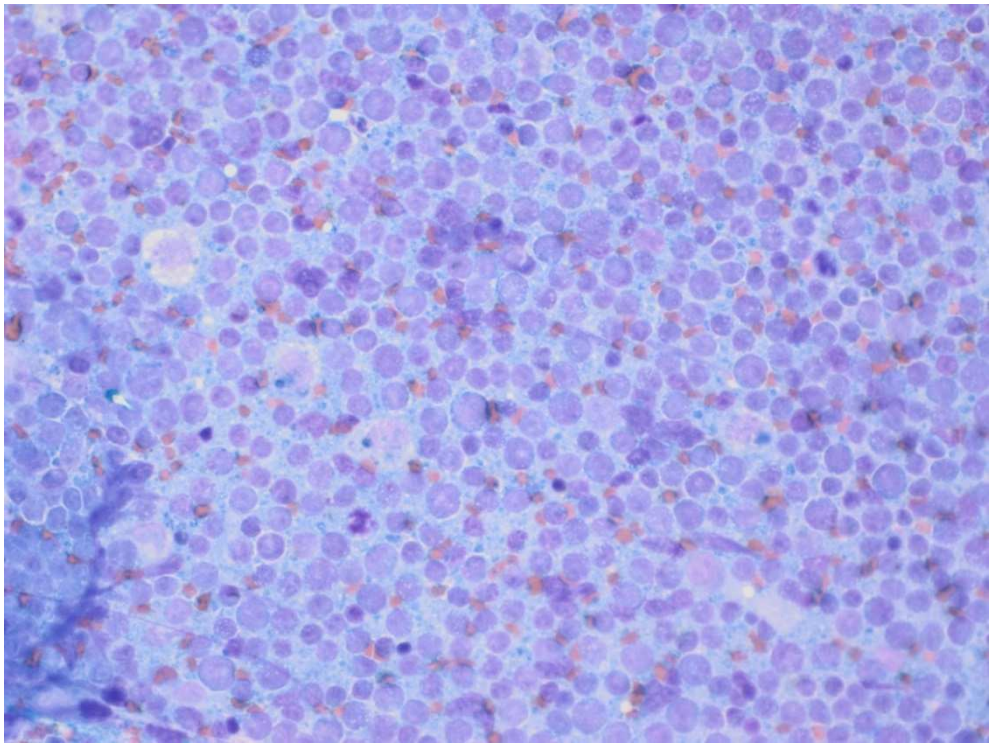


Case no. 19 (sample no. 21): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a monoclonal peak at 108 bp.

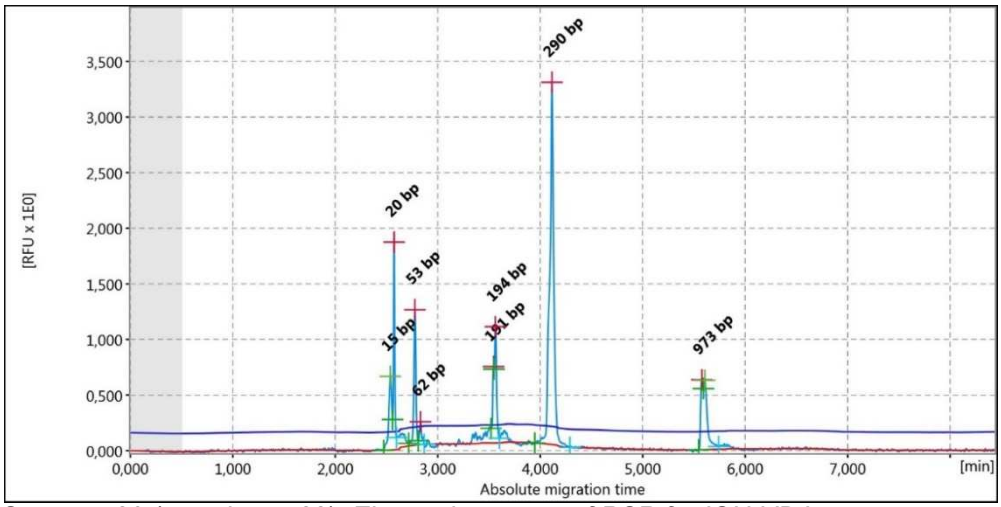


Case no. 19 (sample no. 21): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with a monoclonal peak at 110 bp (IGH-DJ).

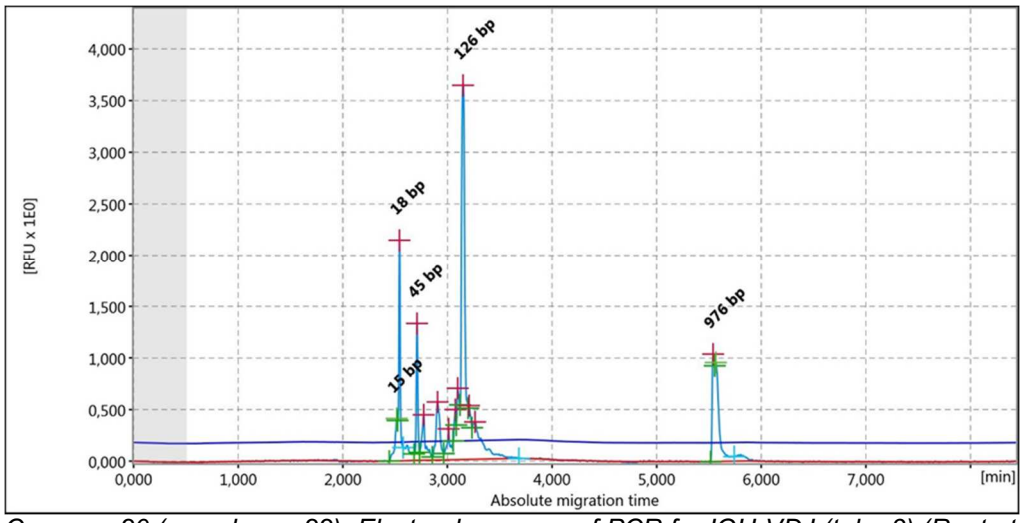
Case 20



Case no. 20 (sample no. 22): Cytology specimen with a C5 classification. Localization: Intestine. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.

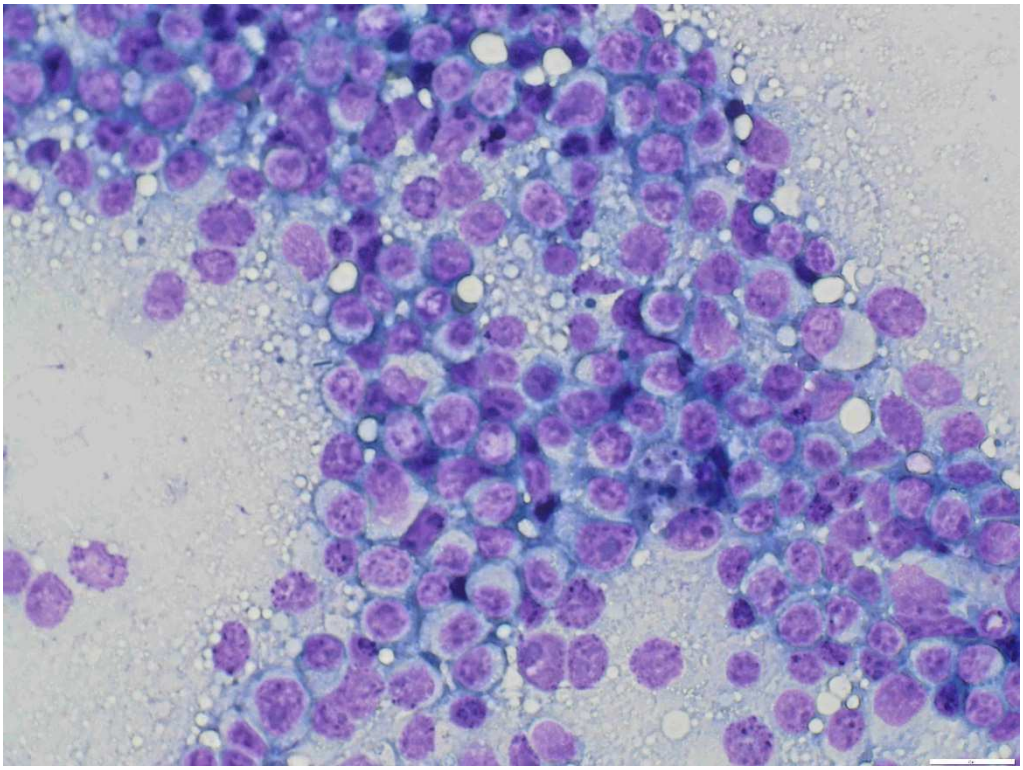


Case no. 20 (sample no. 22): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 290 bp.

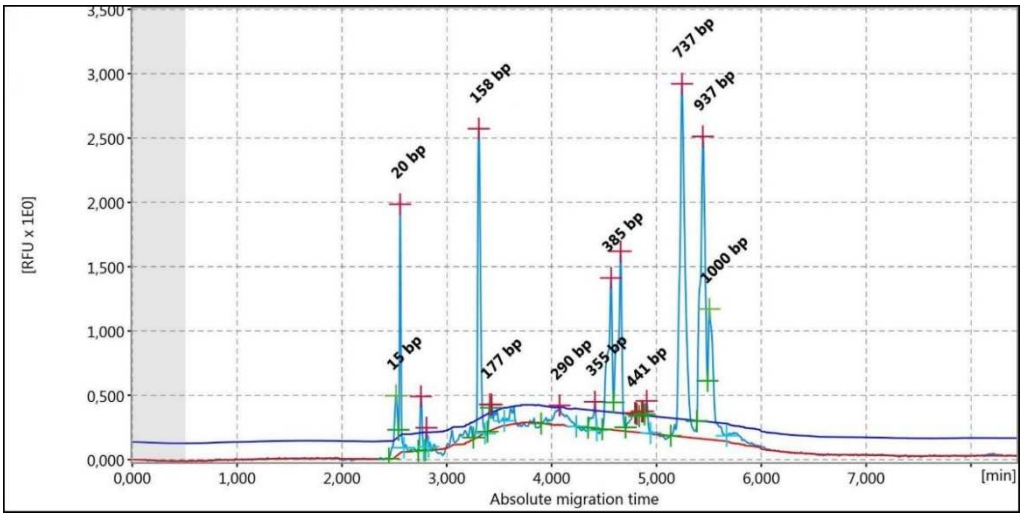


Case no. 20 (sample no. 22): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a monoclonal peak at 126 bp in a polyclonal background.

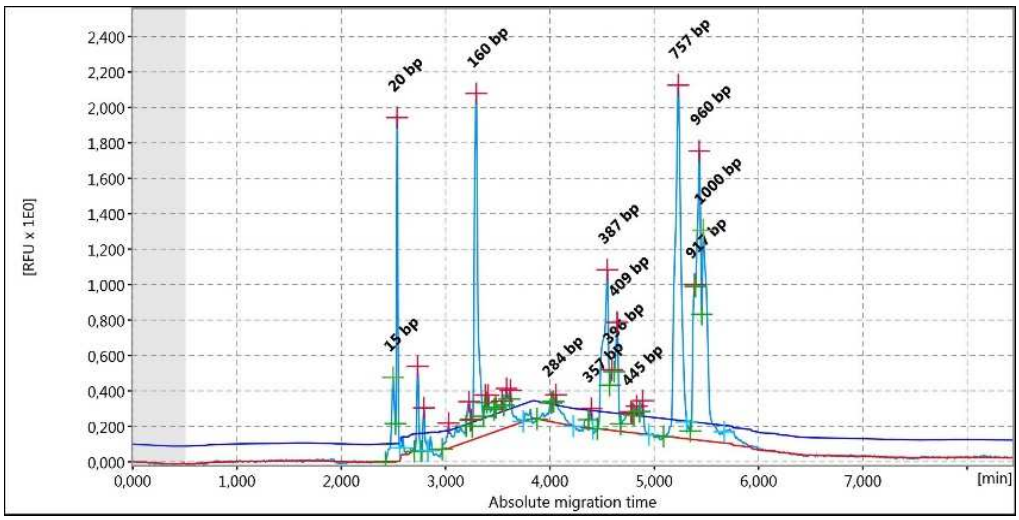
Case 23



Case no. 23 (sample no. 26): Cytology specimen with a C5 classification. Localization: Jejunum. Staining procedure: Romanowsky dip stain (Hemafix®, Biomed). Magnification: 100x.

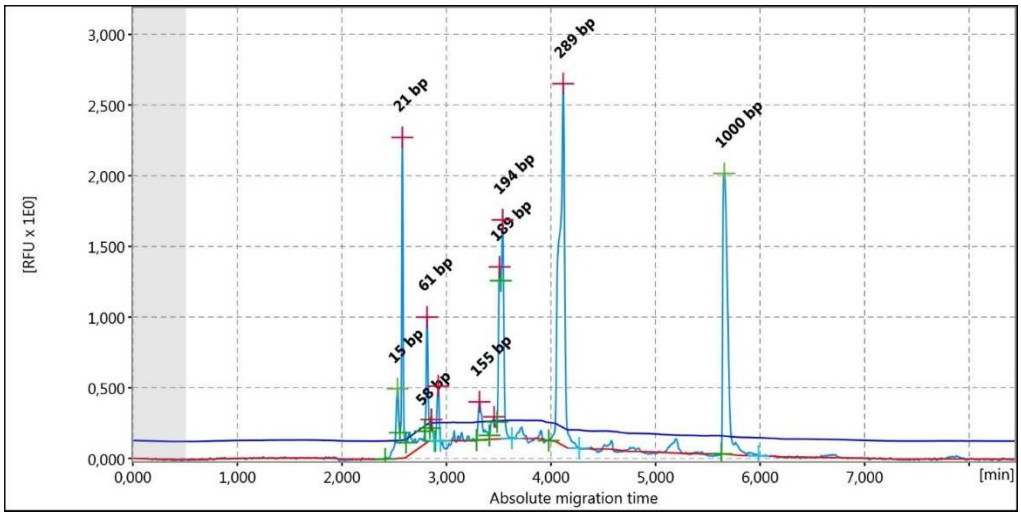


Case no. 23 (sample no. 25): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 158 bp.

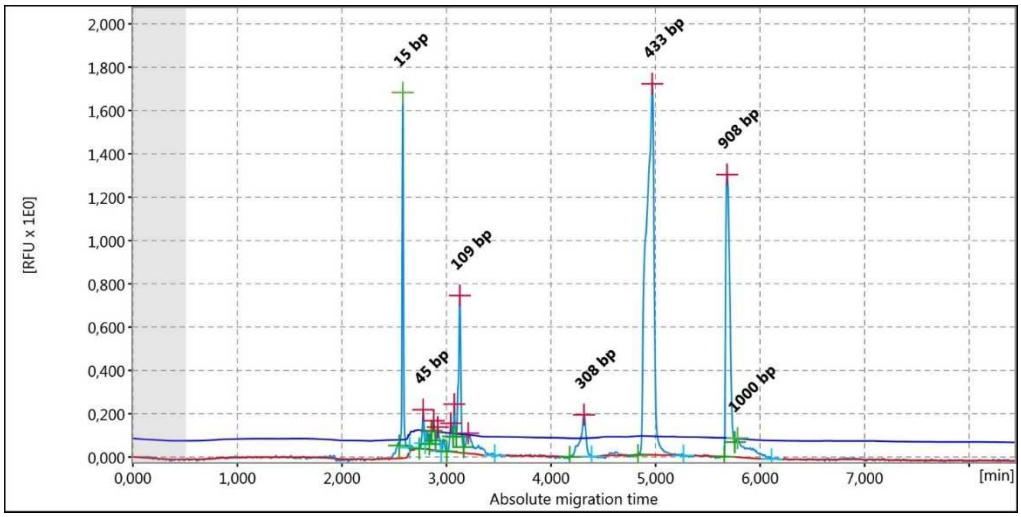


Case no. 23 (sample no. 26): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with a monoclonal peak at 160 bp.

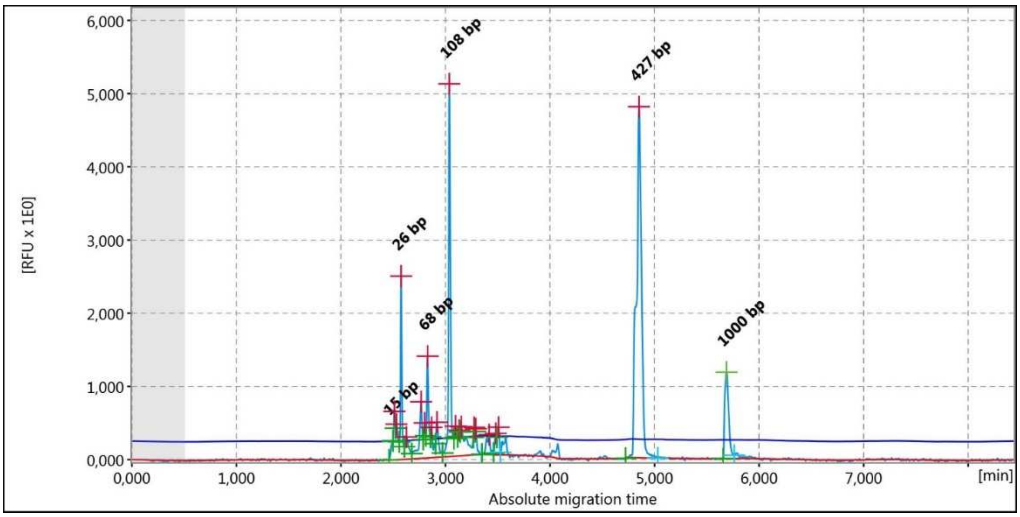
Case 24



Case no. 24 (sample no. 27): Electropherogram of PCR for IGH-VDJ (tube 1) (Mochizuki et al., 2011) with biclonal peaks at 194 and 289 bp.



Case no. 24 (sample no. 27): Electropherogram of PCR for IGH-VDJ (tube 3) (Rout et al., 2019) with a monoclonal peak at 109 bp.



Case no. 24 (sample no. 27): Electropherogram of PCR for IGH-DJ, Kde and IGL (tube 4) (Rout et al., 2019) with a monoclonal peak at 108 bp (IGH-DJ).