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Research paper

Improved clonality analysis based on immunoglobulin kappa locus for canine cutaneous plasmacytoma

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ABSTRACT

Sensitivity of clonality analysis based on immunoglobulin heavy chain (*IGH*) in canine cutaneous plasmacytoma is lower than that in diffuse large B cell lymphoma (DLBCL) because of somatic hypermutation occurring at the *IGH* locus. Therefore, this study aimed to improve the sensitivity of clonality analysis for canine cutaneous plasmacytoma. To achieve this, clonality analysis based on the immunoglobulin kappa chain (*IGK*) locus was established. Sensitivity and specificity were examined in genomic DNA extracted from formalin-fixed paraffin-embedded sections of cutaneous plasmacytomas, DLBCLs, and lymph nodes without lymphoma. Forward primers were designed based on the *IGKV* genes, and reverse primers were designed based on the *IGKJ* genes and kappa deleting element (Kde). Analysis using *IGKV* and *IGKJ* primers demonstrated clonality in 24 of 29 cutaneous plasmacytomas (82.8%), while analysis with primers for *IGKV* and Kde showed clonality in 16 of 29 cases (55.2%). In DLBCL, the *IGKV* and *IGKJ* primer set yielded clonality in 18 of 23 cases (78.3%), and the *IGKV* and Kde primer set yielded 9 of 23 cases (39.1%). No clonal results were obtained from 23 lymph nodes without lymphoma. Sensitivity of the *IGKV* and *IGKJ* primer set was significantly higher than that of the *IGH* primers reported previously. Thus, clonality analysis based on the *IGK* locus can be utilized for canine B cell tumors. In conclusion, clonality testing based on *IGH* and *IGK* may be beneficial as an adjunct tool for diagnosis of canine B cell tumors including cutaneous plasmacytoma.

1. Introduction

Clonality analysis is used as an adjunct diagnosis tool for lymphoproliferative disorders (Werner et al., 2005; Yagihara et al., 2009; Keller et al., 2016). Sensitivity of clonality analysis in canine lymphoma has been reported at approximately 67–91% (Burnett et al., 2003; Thalheim, et al., 2013; Waugh et al., 2016; Takanosu et al., 2018), whereas in humans, sensitivity for B cell tumors is 99% (van Krieken et al., 2007). The reason for this difference may be the target locus for clonality. Although only the immunoglobulin heavy chain (*IGH*) locus has been used in canine clonality analyses published previously, immunoglobulin kappa chain (*IGK*) and immunoglobulin lambda chain (*IGL*) loci are additional targets in humans (van Dongen et al., 2003; Langerak et al., 2012).

In human, the recombination of the immunoglobulin light chain occurs in the following order: kappa chain rearrangement is followed by *IGK* deletion and lambda chain rearrangement during B cell differentiation (van Dongen et al., 2002). The *IGK* light chain locus contains V and J genes for rearrangement, and another element, called kappa deleting element (Kde), is also involved in recombination (van Dongen et al., 2003). Kde is not only rearranged to the V genes but also to an isolated recombination signal sequence in the intron located in the J and C genes (Beishuizen et al., 1994). Recombination including Kde leads to inactivation of the *IGK* allele by deletion of the C gene or the J–C region (van Dongen et al., 2003).

Similar to the human *IGK* locus, canine *IGKV*, *IGKJ*, and Kde could also be targets of clonality analysis. Recombination with Kde results in removal of the *IGK* enhancer, which is thought to be essential for somatic hypermutation; thus, because somatic hypermutation is unlikely to occur at the Kde locus, failure of primers to anneal is unlikely (van

Abbreviations: DLBCL, diffuse large B cell lymphoma; FFPE, formalin-fixed paraffin-embedded; IGH, immunoglobulin heavy chain; IGK, immunoglobulin kappa chain; IGL, immunoglobulin lambda chain; IMGT, International Immunogenetics Information System; Kde, kappa deleting element

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Dongen et al., 2003). In human clonality testing, the *IGK* locus is used as the target in a second step if clonality is not achieved at the *IGH* locus (Langerak et al., 2012). Therefore, canine B cell clonality testing could be improved by targeting the *IGK* locus.

We have previously reported that the sensitivity of clonality analysis based on the *IGH* locus in canine cutaneous plasmacytoma was lower than that in the diffuse large B cell lymphoma (DLBCL), at 41.4% and 91.3%, respectively (Takanosu et al., 2018). This might be due to failure of primers to anneal on the target sequence after somatic hypermutation. Although primer set optimization for canine B cell tumor clonality testing based on the *IGH* locus to improve qualitative sensitivity has been reported (Hwang et al., 2019), the potential of the *IGK* locus as a target for clonality testing remains to be investigated.

Recently, canine antigen receptor loci including *IGK* were bioinformatically annotated (Martin et al., 2018). Canine *IGK* consists of 19 V genes and five J genes; its complete information is published on the International Immunogenetics Information System (IMGT). Information of these would be useful for PCR strategy of clonality analysis based on *IGK*. In the present study, we investigated *IGK* loci including *IGKV*, *IGKJ*, and Kde as targets for canine B cell clonality analysis. This method significantly improves sensitivity in canine cutaneous plasmacytoma compared to the analysis targeting *IGH*.

2. Materials and methods

2.1. Specimens used in this study

Twenty-nine canine cutaneous plasmacytomas, 23 DLBCLs, and 23 lymph nodes without lymphoma were used in this study. Lymph nodes without lymphoma were biopsied for evaluation of metastasis of non-lymphoid malignant tumors (Takanosu et al., 2018). Diagnosis of cutaneous plasmacytomas and DLBCLs and selection of samples were performed as per Takanosu et al. (2018).

2.2. PCR amplification of IGK locus

Locations of each primer on the rearranged *IGKV/IGKJ* and *IGKV/*Kde loci are shown in Fig. 1. Each primer was designed to amplify genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) sections, so that the size of the PCR product, when combined with reverse primers, was < 200 bp. Primer sequences are listed in Fig. 1.

2.3. Primers for IGKV and IGKJ amplification

The sequences of *IGKV* and *IGKJ* genes were obtained from the IMGT database (accession number: IMGT000002). All 19 *IGKV* genes, divided into four subgroups (V2, V3, V4, and V7), or five *IGKJ* genes were aligned using ClustalW (https://www.genome.jp/tools-bin/clustalw). A primer was designed based on the conserved region of the *IGKV2* and *IGKV4* genes and designated as cIGKV_1. The specific primers for three *IGKV* genes, *IGKV2S15*, *IGKV3-3*01*, and *IGKV7-2*01*, which showed no homology with the above genes, were designed and designated as cIGKV_2, cIGKV_3, and cIGK_4. We referred to those four primers as *IGKV* primer group 1. Theoretically, this primer group anneals to every gene belonging to the *IGKV2*, *V3*, *V4*, and *V7* genes. For *IGKJ* genes, a specific primer for each gene was designed and designated as cIGKJ1, cIGKJ2, cIGKJ3, cIGKJ4, and cIGKJ5 (*IGKJ* primers).

2.4. Primers for IGKV and Kde rearrangement

The sequence of canine Kde was obtained using the BLAST search program (National Centre for Biotechnology Information, NCBI) using a human Kde sequence (accession number: X05186 on IMGT) and the canine genome database (CanFam3.1). The putative canine Kde (accession number: NC_006599.3 on NCBI; Position: 37682711–37681815) was identified by alignment with human Kde and showed 66% homology with the human Kde sequence. A reverse primer was designed (cKde) on the putative canine Kde. When the *IGKV* primer group 1 was used as the forward primer combined with cKde, nonspecific products were obtained (data not shown). Thus, other forward primers for the Kde primer were designed on the homology region of *IGKV* genes and designated as cIGK_FR3-1 and cIGK_FR3-2 (*IGKV* primer group 2). The primer cIGK_FR3-1 anneals against five *IGKV2* genes and cIGK_FR3-2 against nine *IGKV2* genes and one *IGKV7* (*IGKV7-2*01*) gene. Theoretically, these two primers anneal against 15 of 19 *IGKV* genes.

2.5. Clonality analysis

Extraction of genomic DNA from FFPE sections and verification of DNA integrity were reported previously (Takanosu et al., 2018). Briefly, genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostic Japan, Tokyo, Japan), and three PCRs were performed for different loci, including *IGH* (130 bp), *ATP7B* (195 bp), and *SOD1* (266 bp). For amplification of *IGKV/IGKJ* rearrangement, the *IGKV* primer group 1 was used as forward primers with *IGKJ* primers as reverse primers. For amplification of *IGKV*/Kde rearrangement, *IGKV* primer group 2 was used as forward primers with cKde primer as the reverse primer. PCR was performed as described by Takanosu et al. (2018) in duplicate to exclude pseudoclonality using 50 ng genomic DNA in a total volume of 20 µL containing 1 × PCR buffer, 0.2 mM deoxynucleotide triphosphates, 0.2 µM of each primer, and 0.5 unit of Taq polymerase (BlendTaq plus, Toyobo Life Science, Tokyo, Japan). Four forward primers (*IGKV* primer group 1) and five reverse primers (*IGKJ* primers) were used in the same tube for amplification of *IGKV/IGKJ* rearrangement. Two forward primers (*IGKV* primer group 2) and one reverse primer cKde were used in the same tube for amplification of *IGKV*/Kde rearrangement. The thermal cycling conditions consisted of 95 °C for 1 min and 35 cycles of 94 °C for 30 s, annealing at the specified temperature (61 °C and 65 °C for *IGKV/IGKJ* and *IGKV*/Kde, respectively) for 20 s, and 72 °C for 30 s. PCR products

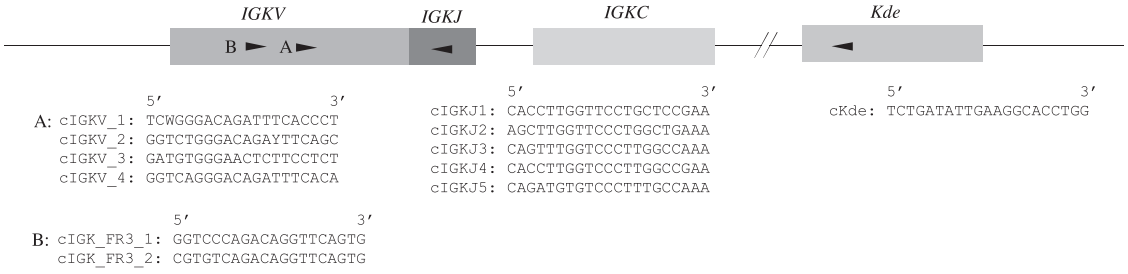

Fig. 1. Schematic representation of *IGK locus*. Locations of primers used in this study are indicated by arrowheads. A and B of primers in *IGKV* indicate primer set group 1 and group 2, respectively. Primer sequences are shown under each diagram of rearrangement of *IGKV, IGKJ* and Kde region. *IGKV*: immunoglobulin kappa V gene; *IGKJ*: immunoglobulin kappa J gene; *IGKC*: immunoglobulin C gene; Kde, kappa deleting element.

were analyzed using a capillary electrophoresis system with a high-resolution cartridge (Qsep-100, BiOptic, New Taipei City, Taiwan).

Interpretation of electrophoretic profiles was performed as described in a previous study (Keller et al., 2016). Clones with one, two, or several distinct and reproducible peak(s) that were two-fold higher than the polyclonal base were defined as clonal (monoclonal, biclonal, or oligoclonal, respectively). Clones with distinct and non-reproducible peak(s) were defined as pseudoclonal. Clones showing a Gaussian distribution were defined as polyclonal. The absence of a PCR product was defined as "no amplification".

2.6. DNA sequencing

Some PCR products amplified from DLBCL were used for DNA sequencing to verify that the PCR products were derived from the *IGK* locus. PCR products were treated with Exo-SAP IT (Thermo Fisher Scientific K.K., Tokyo, Japan) and then sequenced on an ABI 3730xl Analyzer (Applied Biosystems, Foster City, CA) using forward and reverse primers. Because the PCRs were performed by multiplex PCR, it was difficult to identify which of the primers yielded the product, based on the electrophoretic profile only. Thus, each primer was only used once for sequencing analysis.

2.7. Analysis and statistical methods

Fisher's exact test for detection frequency of clonality in cutaneous plasmacytoma was conducted between the novel primers described here and those reported previously (Takanosu et al., 2018). Differences were regarded as statistically significant at $P < 0.05$. When considering multiple tests, the significance level was decreased to $P < 0.0083$ (where $0.05/6 = 0.0083$) according to Bonferroni correction. All statistical analyses were performed using STATA/SE version 13.

3. Results

3.1. Clonality analysis of cutaneous plasmacytomas

The *IGKV* primer group 1 and *IGKJ* primer set yielded PCR products ranging from 110 to 120 bp (Fig. 2). The results of clonality analysis are shown in Table 1. Twenty-four of 29 cutaneous plasmacytomas (82.8%) showed clonality in *IGKV/IGKJ* rearrangement. Of the 29 plasmacytomas, 24 cases showed clonality, two showed polyclonality, and no amplification was observed in three cases for this primer set. The *IGKV*

Table 1
Results of clonality analysis.

Plasmacytoma			DLBCL			LN		
Case	*IGK/IGKJ*	*IGKV*/Kde	Case	*IGKV/IGKJ*	*IGKV*/Kde	Case	*IGKV/IGKJ*	*IGKV*/Kde
1	PS	C	1	P	C	1	P	P
2	C	C	2	C	P	2	P	NA
3	C	C	3	PS	C	3	P	P
4	C	C	4	C	C	4	P	P
5	C	C	5	C	C	5	P	P
6	C	NA	6	C	C	6	P	NA
7	C	NA	7	C	C	7	P	NA
8	C	NA	8	PS	NA	8	P	NA
9	C	NA	9	C	NA	9	P	NA
10	C	NA	10	C	PS	10	P	P
11	C	NA	11	C	NA	11	P	P
12	C	NA	12	C	C	12	P	P
13	C	NA	13	C	NA	13	P	NA
14	C	NA	14	PS	C	14	P	NA
15	C	C	15	P	NA	15	P	NA
16	C	NA	16	C	NA	16	P	NA
17	C	C	17	C	NA	17	P	NA
18	C	NA	18	C	C	18	P	P
19	C	C	19	C	NA	19	NA	NA
20	C	NA	20	C	NA	20	P	NA
21	NA	C	21	C	NA	21	P	NA
22	C	NA	22	C	NA	22	P	NA
23	C	C	23	C	NA	23	P	NA
24	C	C	DLBCL, Diffused large B cell Lymphoma			LN, lymph nodes biopsied for evaluation of metastasis of non-lymphoid tumors		
25	NA	C						
26	PS	C						
27	C	C						
28	C	C						
29	NA	C						

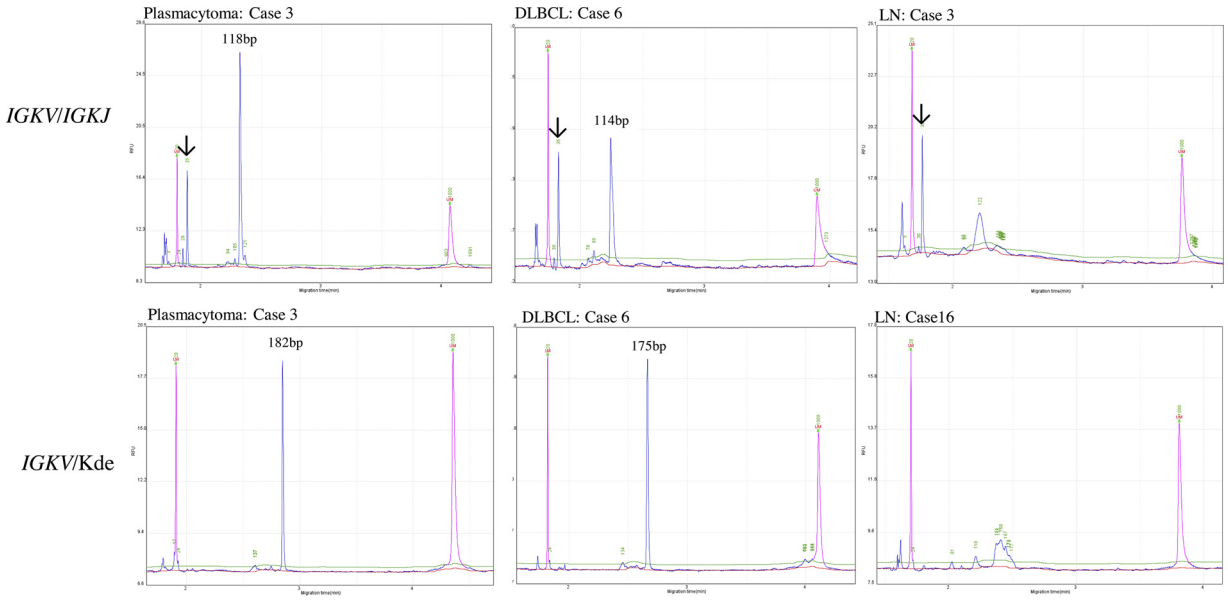

Fig. 2. Representative electrophoretic data from clonality analysis targeting *IGKV/IGKJ* and *IGKV*/Kde. In targeting *IGKV/IGKJ*, 118 base pair (bp) and 114 bp clonal peaks are seen in cutaneous plasmacytoma and DLBCL, respectively. A polyclonal peak is seen in lymph nodes without lymphoma around 120 bp. Arrows indicate a primer-dimer product. In targeting *IGKV*/Kde, 182 bp and 175 bp clonal peaks are seen in cutaneous plasmacytoma and DLBCL, respectively. A polyclonal peak is seen in lymph nodes without lymphoma around 180 bp. The two peaks at the start and end depict the 20 and 1000 bp markers. The vertical axis shows relative fluorescence unit, and the horizontal axis shows migration time.

Table 2
Statistical analysis for clonality in cutaneous plasmacytoma.

Primers used in this study	Primers previously reported[a]	*P*–value	OR (95% CI)
IGKV group 1/*IGKJ1–5* (82.8%)	CB1/CB2 (34.5%)	0.0004*	9.12 (2.66–31.22)
	FR3-1/CB3 (34.5%)	0.0004*	9.12 (2.66–31.22)
	FR2-6/CB2 (20.7%)	< 0.0001*	18.4 (4.93–68.7)
IGKV group 2/Kde (55.2%)	CB1/CB2 (34.5%)	0.1864	2.34 (0.81–6.74)
	FR3-1/CB3 (34.5%)	0.1864	2.34 (0.81–6.74)
	FR2-6/CB2 (20.7%)	0.014	4.72 (1.48–15.03)

P-value and odds ratio (OR) (95% confidence interval [CI]) were assessed using Fisher's exact test. Percentage shows detection rate for each primer.

* $P < 0.0083$.

[a] Takanosu et al., 2018.

primer group 2 and Kde primer set yielded a PCR product from 170 to 180 bp (Fig. 2), and 16 of 29 cutaneous plasmacytomas (55.2%) showed clonality (Table 1). Of the 29 plasmacytomas, 16 cases were clonal, and no amplification was observed in 13 cases for this primer set. All 29 cases showed clonality in any of the primer sets for *IGKJ* or Kde rearrangement.

Fisher's exact test shows that the frequency of detection of clonality in cutaneous plasmacytoma was higher in the *IGKV* primer group 1 and *IGKJ* primer set than in the primer set for *IGH* (Table 2). Contrastingly, the clonality frequency of detection in cutaneous plasmacytoma was not significantly different between the *IGKV* primer group 2 and Kde primer set compared to primers for *IGH*.

3.2. Clonality analysis of lymph nodes with and without lymphoma

Clonality in DLBCLs using the *IGKV* primer group 1/*IGKJ* primers or the *IGKV* primer group 2/Kde was detected in 18 of 23 (78.3%) and nine of 23 (39.1%) cases, respectively (Table 2). Twenty-two of 23 DLBCLs were clonal in any of the primer sets for *IGKJ* or Kde rearrangement (95.7%). In lymph nodes without lymphoma, the polyclonal peak based on the *IGKV* primer group 1/*IGKJ* primers or the *IGKV* primer group 2/Kde yielded around 120 bp or 170 bp, respectively (Fig. 2). No clonality was detected in all 23 lymph nodes without lymphoma in both primer sets, for *IGKJ* and Kde rearrangement (Table 2).

3.3. Sequence analysis of PCR products

Three amplicons for *IGKV/IGKJ* or *IGKV*/Kde rearrangements in DLBCL were analyzed for DNA sequence. In *IGKV/IGKJ* rearrangement, all three cases are matched to *IGKV2* and *IGKJ* genes (Fig.3). In *IGKV*/Kde rearrangement, *IGKV2* genes were recombined with the Kde. These data indicated that PCR products amplified the present study were derived from the *IGK* locus.

4. Discussion

We established clonality analysis based on the *IGK* locus for canine B cell tumors. In our previous study, clonality analysis based on the *IGH* locus showed remarkably low sensitivity in cutaneous plasmacytoma (41.4%) compared to DLBCL (91.3%) (Takanosu et al., 2018). Thus, the aim of this study was to improve sensitivity of clonality analysis for cutaneous plasmacytoma. All 29 canine cutaneous plasmacytomas were detected with either of the two primer sets on the *IGK* locus. Meanwhile, none of 23 lymph nodes without lymphoma showed clonal.

Primers for *IGKV* and *IGKJ* were designed according to *IGK* locus annotation data (Martin et al., 2018), and the Kde primer was designed based on the canine putative Kde locus estimated based on the alignment of the human Kde sequence. The *IGKV* primer group 1 anneals to all 19 *IGKV* genes including *IGKV2*, *V3*, *V4*, and *V7*. When these *IGKV* primers were combined with *IGKJ* primers, sensitivity in cutaneous plasmacytomas and DLBCLs was 82.8% and 78.3%, respectively. In cutaneous plasmacytomas particularly, sensitivity was significantly improved compared to clonality analysis based on the *IGH* locus. Since *IGK* is rearranged in precursor B cells, most mature B cells also have rearranged *IGK* genes. Therefore, *IGK* can be used as another target for clonality analysis in addition to *IGH* in humans (van Dongen et al., 2003). Sensitivity of human clonality analysis for B cell tumors is higher than in dogs because of several targets including *IGH*, *IGK*, and *IGL*, which have been unused in canine B cell clonality analysis so far. To the best of our knowledge, clonality based on *IGK* or *IGL* in dogs has not yet been reported, and thus sensitivity of clonality analysis in dogs is derived from only the *IGH* locus. As in humans, the present study showed that the canine *IGK* locus could be a target for B cell clonality.

It has been previously reported that the reason for the low sensitivity of clonality analysis in canine cutaneous plasmacytoma might be the failure of primers to anneal, caused by somatic hypermutation, at the primer target sequence on *IGH* (Takanosu et al., 2018). Moreover, somatic hypermutation occurs not only on *IGH* but also on *IGK* (van Dongen et al., 2003). However, clonality analysis targeting *IGKV* and *IGKJ* rearrangement showed higher sensitivity in cutaneous plasmacytoma than on *IGH*. This result suggests that the rate of somatic hypermutation on *IGK* is lower than on *IGH* in canine cutaneous plasmacytoma.

When the *IGKV* primer group 1 was combined with the Kde primer, several nonspecific amplicons were obtained in lymph nodes without lymphoma. Thus, two other forward primers located upstream of *IGK* primer group 1 were designed (*IGKV* primer group 2). These two primers anneal against 14 of 15 *IGKV2* genes, which are the main components of *IGKV*, and one *IGKV7* (IGKV7-2*01) gene. Because nonspecific products were not obtained from the above primer combination, the *IGKV* primer group 2 was used as forward primers for Kde rearrangement. Sensitivity of the *IGKV* primer group 2/Kde primer set was 55.2% in cutaneous plasmacytoma and 39.1% in DLBCL. This sensitivity was low compared to the clonality analysis based on the *IGKV* primer group 1/*IGKJ* primer set, suggesting infrequency of the rearrangement of the *IGKV* and Kde loci (van Dongen et al., 2003). In humans, the rearrangement rate of *IGKV* and Kde in adult precursor B cell acute lymphoid leukemia and childhood precursor B cell acute lymphoid leukemia was 35% and 50%, respectively (Beishuizen et al., 1994; Szczepański et al., 1998), which is comparable to that of canine DLBCL and cutaneous plasmacytoma.

It may be important to include detection of Kde rearrangement as well as *IGKV/IGKJ* rearrangement. Although *IGKV* and *IGKJ* rearrangements occur only in immunoglobulin (Ig) kappa positive B cell tumors, Kde rearrangement occurs in all Ig lambda positive B cell tumors to delete the *IGKJ-C* region (van Dongen et al., 2003). It is suggested that the target of Kde rearrangement compensates for the deficiencies of *IGKV/IGKJ* rearrangement, thus increasing the possibility of detecting clonality at the *IGK* locus. In a small-scale immunocytochemical study of canine B cell lymphomas, the lambda chain was found to be preferentially expressed (Sokolowska et al., 2010), which showed that Kde might be frequently rearranged in canine B cell lymphoma. On the contrary, *IGKV/IGKJ* rearrangement was detected in 82.8%, but the detection rate of Kde rearrangement was 55.2% in cutaneous plasmacytoma. Similar to cutaneous plasmacytoma, the detection rate of *IGKV/IGKJ* rearrangement in DLBCLs was higher than that of Kde rearrangement (78.3% and 39.1%, respectively). To elucidate this situation, immunohistochemistry for Ig kappa and lambda chains using a wide range of the B cell tumors is necessary.

Sensitivity of *IGH* clonality analysis is decreased by failure of primers to anneal due to somatic hypermutation. Although framework (FR) 1 and 2 of *IGH* can be other targets for clonality analysis in dogs and cats (Gentilini et al., 2009; Mochizuki et al., 2011), somatic hypermutation also occurs at this region. Furthermore, the size of a PCR

Fig. 3. Sequence analysis of the PCR products amplified from *IGKV/IGKJ* or *IGKV/*Kde rearrangements. Specific gene names are shown following case number, and primers are shown in parentheses. Bold regions indicate forward and reverse primer regions. Underline corresponds to the position of IGKV_1 primer. Arrowheads in Kde rearrangements indicate breakpoint of the *IGJV* gene and Kde region.

product is increased compared to an amplicon from FR3 primers, which may hamper PCR for genomic DNA extracted from FFPE sections. The preferred size of a PCR product for FFPE is less than 200 bp (van Dongen et al., 2003; Langerak et al., 2012). The size of PCR products amplified from primers designed for *IGK* in this study was less than 200 bp, which may be suitable for genomic DNA extracted from both FFPE and fresh or frozen tissues. Therefore, the strategy described here may be useful in a variety of clinical samples.

Clonality analysis based on the *IGK* locus presented here showed high sensitivity to cutaneous plasmacytoma and DLBCL. However, the *IGH* locus may still have benefits compared with the *IGK* locus because recombination of *IGH* occurs earlier than that of *IGK* (van Dongen, et al. 2003). The *IGH* locus would be the first choice for B cell tumors. The *IGK* locus may be suitable for cases not detected by *IGH* targeted analysis for clinical applications.

All 29 cases of cutaneous plasmacytoma but not all 23 cases of DLBCL could be detected in any of the primer sets for the *IGK* locus. Unknown factors that hinder detection still exist in clonality testing. In clonality analysis of B cell tumors in humans, the *IGL* locus is used as a third target (Langerak et al., 2012). *IGL* is rearranged in all Ig lambda positive B cell malignancies and in 5–10% of Ig kappa positive B cell malignancies (van der Burg et al., 2002). Thus, further investigation with consideration of the canine *IGL* locus may be needed to further improve clonality testing for canine cutaneous plasmacytoma.

There are several limitations of this study. First, only cutaneous plasmacytoma was evaluated and multiple myeloma, which is also a plasmacytic tumor, was not studied. The biological behavior of multiple myeloma is different from that of cutaneous plasmacytoma. Therefore, trials on clonality analysis of multiple myeloma based on the *IGK* locus need to be conducted. Second, normal lymph nodes without lymphoma, which were used for evaluating metastasis of some cancers (Takanosu, et al., 2018), were used as the negative control for clonality analysis. It has been reported that some infectious diseases including, ehrlichiosis or leishmaniosis, show clonal expansion of lymphocytes (Avery, 2009). More stringent conditions such as reactive lymph nodes may be necessary to examine specificity of the clonality analysis. Third, *IGKV*–Kde rearrangement was studied, but intron–Kde rearrangement was not. In humans, intron–Kde rearrangement has been detected in 31–55% of B cell malignancies (van Dongen, et al. 2003). The rearrangement rate in canine intron–Kde has not been examined so far. This would lead to failure of detection of clonality in some cases.

5. Conclusion

In clonality analysis based on the *IGK* locus using two primer sets, detection sensitivity was significantly improved in canine cutaneous plasmacytomas compared to the analysis based on *IGH*. These data indicate that clonality analysis based on *IGK* may be another option for diagnosis of canine B cell tumors, including cutaneous plasmacytoma. Using *IGK* locus for cases not detected by the *IGH* locus as a target for clonality analysis would increase the chance to detect clonal populations of lymphocytes. Clonality combined with *IGH* and *IGK* loci should be efficacious in clinical testing. For some cases of lymphoproliferative diseases, definitive diagnosis is difficult to achieve. Thus, improved clonality analysis would facilitate diagnosis of such cases.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

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