NOTE *Clinical Pathology*

Use of Formalin-Fixed Paraffin-Embedded Tissue and Single-Strand Conformation Polymorphism Analysis for Polymerase Chain Reaction of Antigen Receptor Rearrangements in Dogs

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ABSTRACT. PCR for antigen receptor gene rearrangement analysis (PARR) is a new diagnostic method for lymphoid neoplasia. In PARR using formalin-fixed paraffin-embedded tissues (PARR-FFPE), control DNA amplification was successful in only three of five samples. The formalin fixation times of the three samples were shorter than those of the others. Analysis of the formalin fixation time and DNA amplification controls suggested that a formalin fixation time of less than one week is appropriate. Additionally, application of single strand conformation polymorphism (SSCP) for PARR provided clearer results than conventional PARR in 16 unfixed tissues and three FFPE tissues. These results show that PARR-FFPE is viable for tissues with an appropriate formalin fixation time and that application of FFPE and SSCP for PARR are useful for diagnosis and retrospective study of canine lymphoid neoplasia.
KEY WORDS: cancer diagnosis, canine, lymphoma/leukemia, PCR, tumor diagnosis.

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Lymphoid neoplasia is one of the most common malignant tumors in dogs [12]. It can be diagnosed cytologically or histopathologically. Diagnosis is usually straightforward but is difficult in some cases. Polymerase chain reaction (PCR) for antigen receptor gene rearrangement analysis (PARR) was developed for diagnosis of lymphoid neoplasia [1]. Lymphocytes have acquired unique antigen receptor genes of unique lengths and sequences as a result of gene rearrangements. Lymphoid neoplasia is composed of a single population of neoplastic lymphocytes. In PARR, clonal expansion of tumor lymphocytes that contain a rearranged immunoglobulin heavy chain (IgH) gene or T cell receptor gamma (TCRγ) gene can be detected in B-cell or T-cell lymphoid neoplasias, respectively. The PARR is an objective and highly sensitive method, and its usefulness for diagnosis of canine lymphoid neoplasia has recently been proven [1].

Fresh or frozen tissues are usually used for PARR in dogs. In human medicine, formalin-fixed and paraffin-embedded (FFPE) tissues are used [2, 4, 5, 10, 13]. PARR using FFPE tissues (PARR-FFPE) has been recently performed in dogs [11], but in our experience, it does not always succeed. Retrospective PARR studies using preserved FFPE tissues provide useful information about canine lymphoid neoplasia. Therefore, it is important to clarify the characteristics of PARR-FFPE.

In PARR using conventional polyacrylamide gel [1] (referred to as conventional PARR hereafter), it is sometimes difficult to distinguish between a signal obtained from a clonal expansion of neoplastic lymphocytes and that obtained from a non-clonal expansion of non-neoplastic lymphocytes because the amplified DNA is separated only based on its size. Single-strand conformation polymorphism (SSCP) analysis separates different DNA sequences, even if they are the same length, based on their single-strand secondary structure conformations [9]. It has been reported that SSCP improves the degree of diagnostic accuracy of conventional PARR in human medicine [5, 10]. In the present study, we evaluated conventional PARR using FFPE tissues from lymphoma cases and applied SSCP to conventional PARR.

The FFPE tissues for conventional PARR were obtained from the archives at the Laboratory of Veterinary Pathology, Yamaguchi University. They had been fixed in 10% buffered formalin immediately after surgical resection before being embedded in paraffin wax. Four lymphoma tissue samples and one non-neoplastic tissue sample (as a control) were used. These lymphoma tissues had already been pathologically diagnosed, and unfixed samples of these tissues were analyzed using conventional PARR as described previously [1]. Two lymphoma samples were IgH major positive, and the other two samples were TCRγ positive. DNA was extracted from five sections (each section was 4 μm thick and had an area of approximately 1 cm^2) using Takara DEXPAT (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions, and 200 μl of the extracted DNA were concentrated to 15 μl using ethanol precipitation. Conventional PARR was performed as described previously [1]. Briefly, each sample was amplified using two sets of primers (major and minor) for the IgH gene and one set of primers for the TCRγ gene. As a posi-

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tive control for DNA extraction, the conserved region of IgM (Cμ) was also amplified for each sample. The PCR products were electrophoresed using 12% polyacrylamide gel, and the gel was stained with ethidium bromide and visualized with a UV illuminator. If one or more distinct bands were observed, the results were considered to be positive, i.e., one or more tumor lymphocyte clonal expansions were present in the sample. If no bands, some smears, or a ladder of faint bands were observed, the results were considered to be negative. In the two lymphoma samples and the non-neoplastic sample, the Cμ gene was successfully amplified (Fig. 1C, lanes 2, 4 and 6), but this gene was not successfully amplified in the two other lymphoma samples (data not shown). In one lymphoma sample, a distinct band was detected for IgH major, and smears were observed for TCRγ (Fig. 1A and 1B, lane 2) and IgH minor (data not shown). In the other lymphoma sample, a distinct band was detected for TCRγ, and a faint band was observed for IgH major (Fig. 1B and 1A, lane 4) and IgH minor (data not shown). In the non-neoplastic sample, a smear was observed for IgH major, TCRγ (Fig. 1A and 1B, lane 6) and IgH minor (data not shown). These results are consistent with the results of PARR using the unfixed tissue samples (Fig. 1A and 1B, lanes 1, 3 and 5). In contrast, the IgH genes and TCRγ gene could not be amplified in the two lymphoma samples in which the Cμ gene also could not be amplified (data not shown).

All five samples were fixed after resection, but were fixed and embedded at various times. In the case of the three Cμ amplified samples, the formalin fixation times were less than 3 days, and in the case of the two non-Cμ amplified samples, the formalin fixation times were 1 month and unknown. The paraffin-embedding times of the three Cμ amplified samples were 9 months for two of the samples and 18 months for the other. In the case of the two non-Cμ amplified samples, the times were 3 and 28 months. Detection of B cell clonality in FFPE tissue 30 years after fixation has been reported in humans [4]. These findings suggest that long-term formalin fixation inhibits PARR, but not long-term paraffin embedding. To explore the relationship between the formalin fixation time and Cμ gene amplification, extraction of DNA from a complex carcinoma in mammary gland tumor tissue was examined using different formalin fixation times. Six slices, each measuring 15 × 10 × 2 mm, were cut from the carcinoma tissue and then fixed in 10% formalin for 1 or 3 days or 1, 2, 3 or 4 weeks before being embedded in paraffin wax. DNA extraction and Cμ gene amplification were performed as describe above. Cμ gene amplification was detected within 1 week, but faded gradually after 2–3 weeks and was completely gone at 4 weeks (Fig. 2). In human research, it has been reported that IgH gene amplification with good sensitivity is possible for up to 15 days after formalin fixation [2]. Although tissue sample size could affect the duration of DNA preservation in formalin, we suggest that PARR using FFPE samples is viable provided an appropriate fixation time is used (less than 1 week).

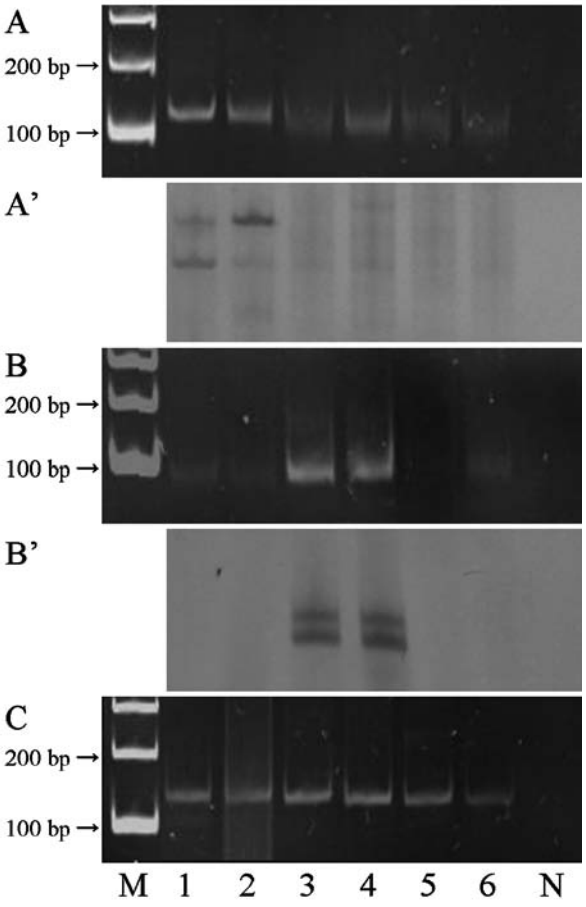

Fig. 1. Conventional PARR and PARR-SSCP for unfixed and FFPE tissues. A & B: Conventional PARR IgH major and TCRγ results, respectively; A' & B': PARR-SSCP IgH major and TCRγ results, respectively; C: Cμ gene. Lanes 1 and 2, 3 and 4 and 5 and 6 are the same tissue. Lane M: 100 bp marker. Lanes 1 and 3: Unfixed lymphoma tissues. Lanes 2 and 4: FFPE lymphoma tissues. Lane 5: Non-neoplastic unfixed tissue. Lane 6: Non-neoplastic FFPE tissue. Lane N: Negative control (without DNA).

To evaluate the application of SSCP analysis for conventional PARR (PARR-SSCP), 16 unfixed tissues were used as samples. DNA was extracted using QIAamp DNA Mini Kits (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA amplification was performed under the same conditions as for conventional PARR. The SSCP procedure was performed as previously described [7]. Briefly, the PCR products were mixed with denaturing loading buffer (95% formamide, 20 mM EDTA and 0.05% bromophenol blue) and boiled for five minutes. The cooled mixtures were loaded onto polyacrylamide gel, electrophoresed at 15 W for 80 min at 20°C and then stained with a Plus One DNA Silver Staining kit (GE Healthcare, Buckinghamshire, England). The method of determining the clonality of PARR-SSCP samples is similar to that for conventional PARR, except that two or more bands are usu-

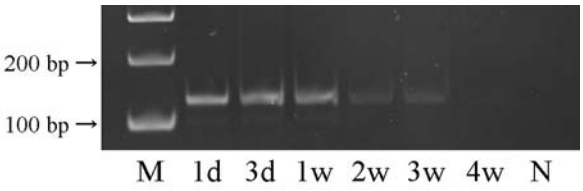

Fig. 2. Cμ gene amplification efficacy after various formalin fixation times. Carcinoma tissue samples of equal size with different formalin fixation times were examined for Cμ gene amplification. Lane M: 100 bp marker. d: Day(s). w: Week(s). Lane N: negative control (without DNA).

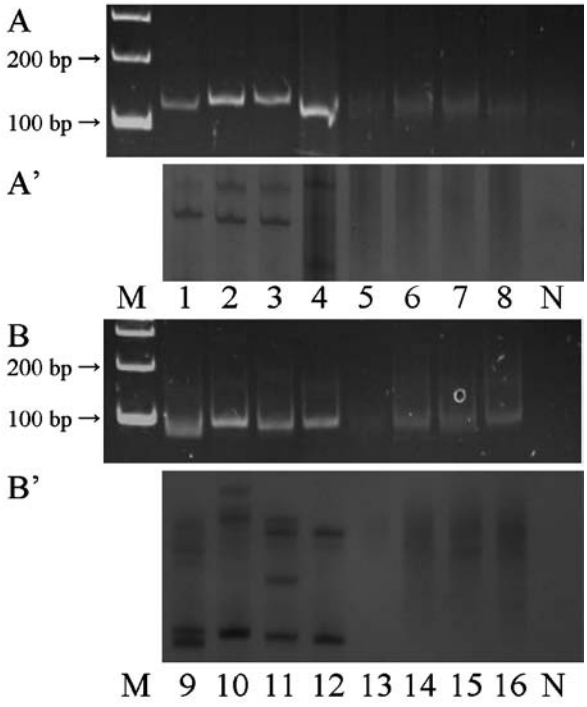

Fig. 3. Comparison of conventional PARR and PARR-SSCP in unfixed tissues. Sixteen specimens that were obtained from clinical cases were examined with conventional PARR (A & B) and PARR-SSCP (A' & B') [Eight specimens for IgH major (A & A') and eight specimens for TCRγ (B & B')]. Lane M: 100 bp marker. Lane N: Negative control (without DNA).

ally observed in positive samples. In the conventional PARR, four samples showed a discrete band for IgH major (Fig. 3A, lanes 1–4), four samples showed a smear or obscure band for IgH major (Fig. 3A, lanes 5–8), four samples showed a discrete band for TCRγ (Fig. 3B, lanes 9–12) and the remaining four samples showed smear or obscure band for TCRγ (Fig. 3B, lanes 13–16). In PARR-SSCP, distinct bands (two or more bands) were also detected in the four IgH major (Fig. 3A', lanes 1–4) and four TCRγ positive samples (Fig. 3B', lanes 9–12). In contrast, the remaining eight samples, which showed smear or obscure band in the conventional PARR, did not show any bands in PARR-SSCP (Fig. 3A', lanes 5–8 and Fig. 3B', lanes 13–16). PARR-SSCP was performed as described above for the three Cμ amplified FFPE tissues. In the FFPE tissues, distinct bands were detected in the PARR-positive samples, but no band was detected in the negative samples (Fig. 1A' and 1B'). Some samples showed more than two bands (ex. Fig. 3B', lane 11). Theoretically, two bands should be observable in PARR-SSCP of a monoclonal lymphoid tumor sample because each double-strand DNA molecule separates into 2 single strands [5]. However, in some reports, several bands have been observed in PARR-SSCP in addition to the target bands, although the cause of this is unknown [3, 6, 8, 10]. Although we could not elucidate the reason for this, our results show that SSCP analysis helps in determination of PARR clonality in samples showing an obscure band in conventional PARR.

To confirm the clonality results obtained in the present study, sequence analyses were performed. The amplified IgH genes of two lymphoma samples (used in Fig. 1A and 1A', lanes 2 and 4) were electrophoresed through 1.5% agarose gel, and the target and obscure bands that they produced were carved out. DNA from the bands was extracted using a QIAEX II Gel Extraction kit (QIAGEN) and cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, U.S.A.). Ten cloned vectors were collected from each sample and sequenced using a BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster, CA, U.S.A.). In the IgH major-positive sample (Fig. 1A and 1A', lane 2), the sequences of the 10 clones were completely identical (121 bases; accession No. AB467330), confirming that PARR-FFPE and PARR-SSCP detected a clonal population containing the rearranged IgH gene. In contrast, in the IgH major-negative (TCRγ positive) sample (Fig. 1A and 1A', lane 4), the IgH sequences of three clones were identical, but those of the remaining seven clones were different from each other (98 to 144 bases; accession No. AB467331-AB467338), suggesting that the obscure band observed in PARR-FFPE (Fig. 1A, lane 4) did not indicate a clonal population containing the rearranged IgH gene. These results confirm the PARR-FFPE and PARR-SSCP results obtained in this study.

In conclusion, PARR-FFPE is viable when using tissues with an appropriate fixation time, and this suggests that PARR-FFPE can be used to obtain useful information in retrospective studies of canine lymphoid neoplasia. However, because we only analyzed Cμ amplification for examination of the formalin fixation time, the appropriate fixation time should be clarified in a future study. Nevertheless, this study suggests that a formalin fixation time of less than 1 week is appropriate for PARR-FFPE. It also suggests that PARR-SSCP provides clearer results than conventional PARR. PARR-SSCP is a simple, reliable and clear method and ideally should be performed for all samples requiring diagnosis. However, because PARR-SSCP is a more complicated and time-consuming method than conventional PARR, PARR-SSCP may be applied as an additional test in cases where distinction between clonal bands and smears is

difficult after conventional PARR or where the PARR result is inconsistent with the pathological diagnosis and/or clinical findings.

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