Studies on Polymerase Chain Reaction for Antigen Receptor Gene Rearrangement in canine lymphoid neoplasia

(犬リンパ系腫瘍における PCR を用いた抗原受容体遺伝子再構成解析に関する研究)

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CONTENTS

	Pages
General Introduction	3
Chapter 1	7
The Application of the Polymerase Chain Reaction for Analyzing Antigen Receptor Rearrangements to Aid Endoscopic Diagnosis of Canine Alimentary Lymphoma	
Chapter 2	20
The Use of Formalin-Fixed Paraffin-Embedded Tissue and Single-Strand Conformation Polymorphism Analysis for Polymerase Chain Reaction of Antigen Receptor Rearrangements in Dogs	
Conclusion	33
Acknowledgements	37
References	38
Table	43
Figures	44

GENERAL INTRODUCTION

Lymphoid neoplasms are a diverse group of tumors that have in common their origin from lymphoreticular cells. Lymphomas usually arise in lymphoid tissues, such as lymph nodes, spleen, and thymus; however, they may arise in almost any tissue in the body. Lymphoma is one of the most common tumors in the dogs and accounts for approximately 7% to 24% of all canine neoplasia and 83% of all canine hematopoietic malignancies [Vail *et al.*, 2007]. The classification of malignant lymphoma in dogs can be distinguished based on anatomic location, histologic criteria, and immunophenotypic characteristics. The most common anatomic forms of lymphoma, in order of decreasing prevalence, are the multicentric (~80%), alimentary (~7%), cutaneous (~6%), mediastinal (~5%), and miscellaneous extranodal sites (central nervous system, bone, heart, nasal cavity, and primary ocular locations) [Vail *et al.*, 2005].

Diagnosis of canine lymphoma relies on the cytologic or histologic examination of the affected tissue. The diagnosis is often straightforward. For instance, it is easy to take enough amounts of cells for cytologic examination from superficial large lymph node in multicentric lymphoma. However, it is difficult for clinicians to biopsy according to the anatomical location of lymphoma. Furthermore, it is difficult for pathologists to diagnosis in some cases, where biopsy samples contain large numbers of reactive lymphocytes, or do not fully represent the lesion. Especially, alimentary lymphoma is difficult to diagnose by endoscopy in both causes. Biopsy samples are often obtained from the intestine, where legions of lymphoma cannot be discriminated from inflammation in the endoscopic view. Furthermore, the samples obtained by the endoscopy are usually small, and contain large number of lymphocytes and plasmacytes. Therefore, more sensitive and objective diagnostic examination is desirable for detecting lymphoid malignancies in dogs.

The polymerase chain reaction (PCR) for antigen receptor gene rearrangement analysis (PARR) was developed for the diagnosis of lymphoid neoplasia [Burnett *et al.*, 2003, Valli *et al.*, 2006]. Because lymphoid neoplasias, including lymphoma, lymphocytic leukemia, and myeloma, are clonal expansions of lymphocytes, each particular neoplasm contains DNA regions that are unique in both lengths and sequences. The variable region of both immunoglobulin (Ig) and T-cell receptor (TCR) genes encodes the antigen-binding region of the respective receptors and contains the majority of the unique sequence. In B cells, the variable region of the Ig is produced through the rearrangement of V, D, and J genes. In T cells, the variable region of the TCR gene is produced through rearrangement of V and J genes. During the process of the rearrangement, five to 20 random nucleotides are added between each gene segment, creating genes with a broad diversity in sequence and length. In PARR, the variable region is amplified by primers specific for conserved regions of V and J genes, followed by size separation of the PCR products. The target genes were immunoglobulin heavy chain (IgH) and TCRγ genes in B and T cells, respectively. TCRγ gene is rearranged in both αβ and γδ T cells in the early mature process, though it is not expressed in αβ T cell, which is major population of T cells in dogs. Furthermore, studies in the literatures suggested that in humans TCRγ rearrangements may be easier to detect, and may also be a more sensitive measure of clonality in comparison to TCRβ rearrangements [Diss *et al.*, 1995, Theodorou *et al.*, 1996, Theodorou *et al.*, 1994]. 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.

PARR is highly sensitive and specific method for diagnosis of lymphoid neoplasia. It

has been reported that PARR was positive in 91% of the 77 dogs with lymphoid malignancy, and was negative in 96% of the 24 dogs with non-lymphoid malignancy [Burnett *et al.*, 2003]. In human studies, PARR are similarly estimated to be approximately 70% to 90% sensitive and 95% specific [Arber, 2000, Arber *et al.*, 2001, Bagg *et al.*, 2002, Diss *et al.*, 1995]. Furthermore, it has been reported that PARR could be detected when 0.1-10% of the DNA was derived from neoplastic cells, depending on the source tissue [Burnett *et al.*, 2003]. It means that PARR can detect a small number of neoplastic lymphocytes in biopsy specimens that contains numerous reactive lymphocytes. However, in PARR, 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.

From these backgrounds, a series of the present studies were carried out to clarify the property of PARR for clinical usage against the canine lymphoid neoplasia. In chapter 1, I evaluated the usefulness of PARR using endoscopic biopsy specimens for the diagnosis of alimentary lymphoma. In chapter 2, I evaluated the possibility of PARR using formalin-fixed paraffin-embedded tissues for retrospective PARR studies. Furthermore, I applied single-strand conformation polymorphism analysis for improvement of diagnostic accuracy of PARR.

Chapter 1

The Application of the Polymerase Chain Reaction for Analyzing Antigen Receptor Rearrangements to Aid Endoscopic Diagnosis of Canine Alimentary Lymphoma

SUMMARY

I evaluated the usefulness of the polymerase chain reaction for antigen receptor gene rearrangement analysis (PARR) of endoscopic biopsy specimens for the diagnosis of canine alimentary lymphoma. Two endoscopic biopsy specimens were obtained from the lesions of 78 dogs with gastrointestinal symptoms. One specimen was histopathologically examined by a pathologist, and the other was analyzed by PARR. All samples were categorized into three groups [lymphoma (n=4), adenocarcinoma (n=5) and enteritis groups (n=69)], based on their histopathological diagnosis. In the lymphoma group, one case was IgH major positive, and three cases were TCRγ positive, representing clonal expansion of B- and T-cells, respectively. In the PARR of the adenocarcinoma group, all cases were negative. In the enteritis group, six cases were TCRγ positive. Two of the six TCRγ- positive enteritis cases were cytologically diagnosed as lymphoma by fine needle aspiration during a laparotomy. In the enteritis group, survival times were compared between the TCRγ- positive and negative cases. The overall survival time of the TCRγ- positive enteritis cases was significantly shorter than that of the TCRγ- negative enteritis cases according to a log-rank test ($p<0.0001$). With regard to other conditions, such as age, clinical signs, and serum albumin concentration, there were no significant differences between the TCRγ- positive and negative enteritis cases. In conclusion, PARR is able to detect alimentary lymphoma and latent alimentary lymphoma, which cannot be histopathologically diagnosed using endoscopic biopsy specimens. Furthermore, a TCRγ- positive result in PARR may imply poor prognosis.

INTRODUCTION

Lymphoma is one of the most common tumors in dogs. Alimentary lymphoma usually accounts for 5% to 7% of all canine lymphomas [Theilen *et al.*, 1987]. Dogs with alimentary lymphoma show nonspecific gastrointestinal signs, such as vomiting, diarrhea, anorexia, weight loss, hypoproteinemia, and malabsorption. Alimentary lymphoma usually occurs multifocally and/or diffusely throughout the submucosa and lamina propria of the intestine, especially in the small intestine, with frequent superficial ulceration and occasional transmural infiltration of the serosa [Vail *et al.*, 2007]. Although alimentary lymphomas that involve the formation of a mass can be detected by diagnostic imaging, it is difficult to detect diffuse alimentary lymphomas that do not involve a mass. Lymphocytic-plasmacytic inflammation can occur adjacent to or distant from the primary tumor, and distinguishing between alimentary lymphoma and enteritis, especially lymphocytic-plasmacytic enteritis (LPE), can be difficult [Couto *et al.*, 1989, Vail *et al.*, 2007]. Additionally, there is interobserver variation among histopathologic evaluations of intestinal tissues [Willard *et al.*, 2002]. Therefore, a full thickness biopsy of the intestine is desirable to make an accurate diagnosis; however, dogs that are suspected of having alimentary lymphoma are usually in a poor condition and are unlikely to survive a long period of anesthesia, and laparotomic damage may also occur in some cases. There is also the risk of intestinal perforations at the laparotomic biopsy site in dogs with hypoalbuminemia or intensive infiltration of inflammatory cells and/or lymphoma cells into the intestine [Ralphs *et al.*, 2003]. In contrast, the endoscopic biopsy is less invasive than the laparotomy, but is considered to be inadequate because only a small and superficial (mucosa) specimen can be obtained [Kleinschmidt *et al.*, 2006, Vail *et al.*, 2007].

Recently, the polymerase chain reaction (PCR) for antigen receptor gene rearrangement analysis (PARR) was developed for the diagnosis of lymphoid neoplasia [Burnett *et al.*, 2003, Valli *et al.*, 2006]. Lymphocytes have acquired a unique antigen receptor gene, which is of a unique length and sequence, by gene rearrangement. 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 a T cell receptor gamma (TCRγ) gene can be detected in B-cell and T-cell lymphoid neoplasias, respectively. PARR is an objective and highly sensitive method, and its usefulness for the diagnosis of canine lymphoid neoplasia has recently been proven [Burnett *et al.*, 2003, Keller *et al.*, 2004, Lana *et al.*, 2006, Valli *et al.*, 2006]. In this study, I evaluated the usefulness of PARR using endoscopic biopsy specimens for the diagnosis of alimentary lymphoma.

MATERIALS AND METHODS

Tissue specimens

From October 2003 to October 2005, 78 dogs that suffered from gastrointestinal symptoms such as diarrhea or vomiting and that had been examined by endoscopy in Yamaguchi University Animal Medical Center, Nihon University Animal Medical Center and other five private animal hospitals, were included in this study. Two endoscopic biopsy specimens were obtained from each lesion with endoscopic biopsy forceps. One specimen was fixed in 10% buffered formalin and evaluated histopathologically in the hospital's respective pathological laboratories or commercial laboratories. All the specimens were classified into three groups (lymphoma, adenocarcinoma, and enteritis groups), based on their histopathological diagnosis. Another specimen was placed in 200 μl of saline and stored at -20 °C until use.

PARR

The endoscopic biopsy specimen was homogenized, and DNA was extracted using QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. PARR was performed as described previously [Burnett *et al.*, 2003]. Briefly, each sample was amplified by two sets of primers (major and minor) for the IgH gene and one set of primers for the TCRγ gene. As a positive control for the DNA extraction process, the constant 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 an 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. The PARR result, which was obtained from a pathologically representative lesion, was considered as the clonality result of each dog in the statistical analysis, if histopathological specimens were obtained from more than one lesions of each dog.

Statistical analysis

In the enteritis group, the overall survival time, age, and serum albumin concentration were compared between the TCRγ- positive and negative cases. Overall survival time was evaluated with the Kaplan-Meier method and the log-rank test. The age and albumin concentration were examined for normality by the D'Agostino and Pearson normality test and compared by the Mann-Whitney U test. The clinical signs were compared by the Fisher's exact test. A p value < 0.05 was considered statistically significant. Calculations were performed with statistical software (Prism 5, GraphPad Software Inc., San Diego, CA).

RESULTS

Dogs

The median age of the 78 dogs was 6.0 years (range: seven months to 16 years), and their median weight was 6.8 kg (range: 2.3 to 53 kg). Forty-seven dogs were male (31 entire, 16 neutered) and 31 were female (14 entire, 17 neutered). Seventy dogs representing 27 pure breeds were affected, as were eight dogs of mixed breed. The most commonly affected breeds were Miniature Dachshund (n=14), Shih Tzu (n=5), West Highland White Terrier (n=4), Pembroke Welsh Corgi (n=4), and Shiba (n=4). The clinical signs observed were vomiting (n=50), diarrhea (n=40), anorexia (n=32), lethargy (n=24), and effusion (n=4). The mean ± standard deviation of the albumin concentrations measured in 68 dogs in the seven days before the endoscopy were 2.5 ± 0.9 g/dl.

Histopathological diagnoses

The endoscopic specimens were examined histopathologically by pathologists. There were cases of lymphoma (n=2); cases suggestive of lymphoma (n=2); and cases of adenocarcinoma (n=5), LPE (n=27), chronic enteritis (n=39), haemorrhagic enteritis (n=2), and polyp (n=1). These were classified into three groups: the lymphoma, adenocarcinoma, and enteritis groups. The lymphoma group included the lymphoma and suggestive of lymphoma cases. The adenocarcinoma group included only the adenocarcinoma cases. The enteritis group included the LPE, chronic enteritis, haemorrhagic enteritis, and polyp cases.

PARR

Based on histopathological observations, specimens obtained from the stomach (n=8), duodenum (n=65), and colon (n=5) were determined to represent the main lesion of each dog, and the PARR results of the main lesion were considered to represent the clonality result of each case. In PARR, Cμ as a control for the DNA extraction process was successfully amplified in all cases, confirming the DNA extraction of the specimens. IgH major was positive in one lymphoma case. TCRγ was positive in three lymphoma cases and six enteritis cases. IgH minor was negative in all cases, and there were no multi-positive cases. Therefore, all the lymphoma cases were positive according to PARR. Importantly, two of the six TCRγ- positive enteritis cases, which were not diagnosed as lymphoma by the endoscopic specimens, were cytologically diagnosed as lymphoma by fine needle aspiration during a laparotomy. In the other four TCRγ-positive enteritis cases, neither laparotomy nor autopsy was performed. All the adenocarcinoma cases produced negative PARR results.

Comparison of histopathology and PARR

Follow-up information was available in 55 cases. All the lymphoma cases and the two enteritis cases that had been cytologically diagnosed as lymphoma were treated with multidrug (CHOP-based protocol) and/or lomustine chemotherapy. One TCRγ-positive enteritis case, which was not diagnosed as lymphoma by histopathology, was also treated with multidrug chemotherapy but died 19 days after endoscopy. The other enteritis cases were treated with dietary change, metronidazole, sulfasalazine, and/or immunosuppressive therapy, including prednisolone, azathioprine, and cyclosporine.

The overall survival time of the TCRγ- positive enteritis cases was compared to that

of the TCRγ- negative enteritis cases. Follow-up information was available in six TCRγ-positive and 45 TCRγ- negative enteritis cases. The overall survival time of the TCRγ-positive enteritis cases was significantly shorter than that of the TCRγ- negative enteritis cases according to the log-rank test ($p<0.0001$) (Fig. 1). The two lymphoma cases of the enteritis group, which had been cytologically diagnosed as lymphoma at their concurrent laparotomy, died at 34 and 153 days, respectively (Fig. 1). With regard to the other conditions, including age, clinical symptoms, and serum albumin concentration, there were no significant differences between the TCRγ- positive and -negative enteritis cases (Table 1).

DISCUSSION

In all the cases of the lymphoma group, the clonalities of the antigen receptor genes were detected with PARR using endoscopic biopsy specimens. The sensitivity of PARR for histopathologic diagnosis was 100%, although the sample number was limited. PARR has been shown to be a highly sensitive method, but sometimes causes false negative results, if the clonality is not detected with the primers used, if the lymphoid neoplasia originates from natural killer cells, or if the malignant cells are present in numbers too low to be detected [Avery *et al.*, 2004]. All the adenocarcinoma cases were produced negative PARR results. This confirms that PARR does not detect tumors except lymphoid neoplasias in spite of tumor cells containing the clonal gene, because the antigen receptor genes of the tumors are not rearranged.

The clonality of TCRγ was detected in six enteritis cases. Lymphoma was diagnosed cytologically during laparotomy in two of the six cases. This suggests that PARR can detect latent lymphoma cases, which can not be histopathologically diagnosed lymphoma using endoscopic biopsy specimens. In this study, the pathologists could not identify the specimens as lymphoma. This may be result from subtle differences between the biopsy specimens, because they were taken from close but not identical lesions. Moreover, it has been reported that interobserver variation and the quality of endoscopic biopsy specimens influences histopathological outcome [Willard *et al.*, 2002, Willard *et al.*, 2008]. Since the endoscopic biopsy specimens were taken by several operators and evaluated by several pathologists, this may have influenced the histopathological diagnoses obtained in this study. Recently, The World Small Animal Veterinary Association Gastrointestinal Standardization Group standardized the method for the diagnosis of gastrointestinal inflammation in endoscopic biopsy specimens from

dogs and cats [Day *et al.*, 2008]. It is important to use the protocol in future studies.

Lymphoma was not diagnosed in the remaining four cases. It is unknown whether they were true lymphomas, because they were not examined by laparotomy or autopsy. Recently, we have experienced a canine case, which was histopathologically diagnosed as LPE according to an endoscopic biopsy specimen but was TCRγ- positive according to PARR. The dog did not respond to treatment (metronidazole, prednisolone, and azathioprine), and was confirmed as having LPE by autopsy (Kaneko and Okuda, unpublished observations). False positive PARR results have been reported for some infectious diseases, such as ehrlichiosis [Burnett *et al.*, 2003] and Lyme disease. Therefore, the PARR results of the four TCRγ- positive enteritis cases could have been false positives. In order to clarify this, I cloned the PCR-amplified products into a plasmid vector and performed sequence analysis. However, I could not obtain their sequence, probably due to their short length.

In the enteritis group, the overall survival times were compared between the TCRγ-positive and negative cases. The survival time of the TCRγ- positive enteritis cases was significantly shorter than that of TCRγ- negative enteritis cases ($p<0.0001$) (Fig. 1). The survival times of the two TCRγ- positive cases that were diagnosed as lymphoma by laparotomy were 34 and 153 days, respectively (Fig. 1), suggesting that the two cases were unlikely to be a major cause of the poor survival time of the TCRγ- positive cases. This result suggests that a TCRγ- positive result in the enteritis cases implies poor prognosis. Other factors were also compared between TCRγ- positive and negative enteritis cases (Table 1). Age, clinical symptoms, and serum albumin concentration were not significantly associated with TCRγ clonality, although some parameters, including serum albumin concentration and weight loss have been reported as prognostic factors

of inflammatory bowel disease and LPE [Craven *et al.*, 2004, Ohno *et al.*, 2006]. These findings suggest that TCRγ clonality was the only negative factor for survival time in the enteritis group. It has been reported that neoplastic lymphocytes of canine alimentary lymphoma usually originate from T cells [Miura *et al.*, 2004] and that canine T-cell lymphoma cases are associated with shorter survival durations [Ruslander *et al.*, 1997, Teske *et al.*, 1994]. However, the PARR results of the four TCRγ- positive enteritis cases could have been false positives as described above. I could not elucidate why TCRγ- false positive enteritis cases would have a poor prognosis (if they exist).

LPE can occur adjacent to or distant from the primary tumor [Couto *et al.*, 1989, Vail *et al.*, 2007]. In addition, it is difficult to diagnosis lymphoma histopathologically when only a small number of malignant lymphocytes are present among the normal lymphocytes and plasma cells. In contrast, PARR is able to detect the clonality when 0.1-10% of the DNA is derived from neoplastic cells [Burnett *et al.*, 2003]. Furthermore, LPE has been described in basenjis that subsequently developed alimentary lymphoma [Breitschwerdt *et al.*, 1982]. Therefore, the TCRγ- positive results in the enteritis cases may imply that PARR is able to detect latent lymphoma or prelymphomatous changes in endoscopic biopsy specimens. If a clonal TCRγ gene is detected in endoscopic biopsy specimens, even if the specimens were histopathologically diagnosed as enteritis, additional examinations, including laparotomy or laparoscopy, are recommended to search for latent alimentary lymphoma.

In conclusion, in this study PARR was able to detect alimentary lymphoma and latent alimentary lymphoma, which cannot be diagnosed histopathologically using an endoscopic biopsy. Furthermore, these results suggest that a clonal TCRγ gene implies poor prognosis and lymphoma or prelymphomatous change in dogs suffering from

enteritis. However, due to the small numbers of lymphoma and PARR positive cases, the lack of pathological standardization, and the lack of uniformity of treatment, further studies are necessary to confirm the relationship between TCRγ clonality and prognosis.

Chapter 2

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

SUMMARY

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 control DNA amplification suggested that a formalin-fixation time of one week or less is appropriate. Additionally, application of 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 in tissues with an appropriate formalin-fixation time and that the application of FFPE and SSCP for PARR is useful for diagnosis and retrospective studies of canine lymphoid neoplasia.

INTRODUCTION

Lymphoid neoplasia is one of the most common malignant tumors in dogs [Vail *et al.*, 2001]. It can be diagnosed cytologically or histopathologically. Diagnosis is usually straightforward but is difficult in some cases. The polymerase chain reaction (PCR) for antigen receptor gene rearrangement analysis (PARR) was developed for the diagnosis of lymphoid neoplasia [Burnett *et al.*, 2003]. Lymphocytes have acquired unique antigen receptor genes, which are of unique lengths and sequences, by 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 a T-cell receptor gamma (TCRγ) gene can be detected in B-cell or T-cell lymphoid neoplasias, respectively. The PARR is objective and highly sensitive method, and its usefulness for the diagnosis of canine lymphoid neoplasia has recently been proven [Burnett *et al.*, 2003].

Fresh or frozen tissues are usually used for the PARR in dogs. In human medicine, formalin-fixed and paraffin-embedded (FFPE) tissues are used [Camilleri-Broet *et al.*, 2000, Diss *et al.*, 1994, Han *et al.*, 2004, Signoretti *et al.*, 1994, Wan *et al.*, 1990]. PARR using FFPE tissues (PARR-FFPE) has been recently performed in dogs [Valli *et al.*, 2006], but in my 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 [Burnett *et al.*, 2003] (referred to as conventional PARR hereafter), it is sometimes difficult to distinguish between a signal obtained from a clonal expansion of neoplastic lymphocytes with that 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 have the same length, based on their single-strand secondary structure conformations [Orita *et al.*, 1989]. It has been reported that SSCP improves the degree of diagnostic accuracy of conventional PARR in human medicine [Han *et al.*, 2004, Signoretti *et al.*, 1999]. In this study, I evaluated conventional PARR using FFPE tissues from canine lymphoma cases and applied SSCP to conventional PARR.

MATERIALS AND METHODS

Specimens

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 negative control) were used. These lymphoma tissues had already been pathologically diagnosed, and their unfixed samples were analyzed using conventional PARR in chapter 1. Two lymphoma samples were IgH major positive, and the other two samples were TCRγ positive. Their formalin-fixation times were 3 days or less in three samples, 1 month in one sample, and unknown in one sample. Their paraffin-embedded times were from 3 to 28 months.

To explore the relationship between the formalin-fixation time and Cμ gene amplification, a complex carcinoma of mammary gland tumor tissue was examined for DNA extraction with different formalin-fixation times. Six slices, each 15 x 10 x 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.

To evaluate the application of SSCP analysis for conventional PARR, 16 unfixed tissues and three FFPE tissues were used as samples. The FFPE tissues were three of the samples used at the above PARR using FFPE.

DNA extraction

In the FFPE samples, 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. In the unfixed samples, DNA was extracted using QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

Conventional PARR and PARR-SSCP

Conventional PARR was performed as described in chapter 1. Briefly, each sample was amplified by two sets of primers (major and minor) for the IgH gene and one set of primers for the TCRγ gene. As a positive 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 an 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 was observed, the results were considered to be negative.

In PARR-SSCP, the SSCP procedure was performed as previously described [Kaneko *et al.*, 2005]. Briefly, the PCR products were mixed with the same amount of denaturing loading buffer (95% formamide, 20 mM EDTA, and 0.05% bromophenol blue) and boiled for five minutes. Five μl of the cooled mixtures were loaded onto 12.5% polyacrylamide gel, electrophoresed at 15 W for 80 minutes at 20°C, and then stained with Plus One DNA Silver Staining Kit (GE Healthcare, Buckinghamshire, England). The method for determining the clonality of PARR-SSCP samples is similar to that for conventional PARR, except that two or more bands are usually observed in positive

sample.

Sequence analyses

The amplified IgH genes of two lymphoma samples were electrophoresed through 1.5% agarose gel, and the target and obscure bands that they produced were carve out. DNA from the bands was extracted using QIAEX II Gel Extraction Kit (QIAGEN) and cloned into TOPO TA cloning vector (Invitrogen, Carlsbad, CA). Ten cloned vectors were collected from each sample and sequenced using BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster, CA).

RESULTS

Conventional PARR using FFPE tissues

The FFPE tissues of four lymphoma samples and one non-neoplastic sample were used for conventional PARR. In the two lymphoma samples and the non-neoplastic sample, the Cμ gene was successfully amplified (Fig 2C, lanes 2, 4, and 6), but this was not the case 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. 2A and 2B, lane 2) and IgH minor (data not shown). In the other lymphoma sample, a distinct band was detected for TCRγ, and faint bands were observed for IgH major (Fig. 2A and 2B, lane 4) and IgH minor (data not shown). In the non-neoplastic sample, smears were observed for IgH major, TCRγ (Fig. 2A and 2B, lane 6), and IgH minor (data not shown). These results are consistent with the results of the PARR using the unfixed tissue samples (Fig. 2A and 2B, lanes 1, 3, and 5). In contrast, the IgH genes and the 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 immediately after the resection, but were fixed and embedded at various times. In the three Cμ amplified samples, the formalin-fixation times were 3 days or less, although in 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 in two samples and 18 months in one sample. In the two non Cμ amplified samples, the times were 3 and 28 months.

Analysis of formalin-fixation time and Cμ gene amplification

To explore the relationship between the formalin-fixation time and Cμ gene amplification, a complex carcinoma tissue was fixed in formalin for 1 or 3 days, or 1, 2, 3, or 4 weeks before being embedded in paraffin wax. Cμ gene amplification was detected within 1 week, but faded gradually after 2-3 weeks and had disappeared at 4 weeks (Fig. 3).

Conventional PARR and PARR-SSCP

To evaluate the application of SSCP analysis for conventional PARR, 16 unfixed tissues were used as samples. In the conventional PARR, four samples showed a discrete band for IgH major (Fig. 4A, lanes 1-4), four samples showed smear or obscure band for IgH major (Fig. 4A, lanes 5-8), four samples showed a discrete band for TCRγ (Fig. 4B, lanes 9-12), and the remaining four samples showed smears or obscure bands for TCRγ (Fig. 4B, lanes 13-16). In the PARR-SSCP, distinct bands (two or more bands) were also detected in the four IgH major (Fig. 4A', lanes 1-4) and four TCRγ positive samples (Fig. 4B', lanes 9-12). In contrast, the remaining eight samples, which showed smears or obscure bands in the conventional PARR, did not show any bands in the PARR-SSCP (Fig. 4A', lanes 5-8 and Fig. 4B', lanes 13-16). The PARR-SSCP was also 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. 2A' and 2B').

Sequence analyses

To confirm the clonality results obtained in this study, the amplified IgH genes of two

lymphoma samples were cloned and sequence analyses were performed for each 10 clones. In the IgH major positive sample (Fig. 2A and 2A', lane 2), the sequences of the 10 clones were completely identical (121 bases, accession No. AB467330). In contrast, in the IgH major negative (TCRγ positive) sample (Fig. 2A and 2A', 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).

DISCUSSION

Conventional PARR using FFPE tissues was performed in four lymphoma samples and one non-neoplastic tissue sample. In two lymphoma samples, the Cμ gene was successfully amplified, and one sample was IgH major positive and another was TCRγ positive (Fig. 2). In the non-neoplastic sample, Cμ gene was amplified and PARR was negative. These results are consistent with the results of the PARR using the unfixed tissue samples. However, Cμ gene as well as IgH and TCRγ was not amplified in the two other lymphoma samples (data not shown). In contrast, in PARR using their unfixed tissues, they showed IgH major or TCRγ positive, respectively (data not shown). These five samples had been fixed immediately after the resection, but their fixation and embedding times were variable. The formalin-fixation times of the Cμ amplified samples were less than those of the non Cμ amplified samples. In contrast, the paraffin-embedding times of the Cμ amplified samples were 9 months in two samples and 18 months in one sample. In the non Cμ amplified samples, the times were 3 and 28 months. It seems that there was no relationship between paraffin-embedded times and Cμ amplification. Detection of B cell clonality from FFPE 30 years after fixation has been reported in humans [Diss *et al.*, 1994]. 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, a complex carcinoma of mammary gland tumor tissue was examined for DNA extraction with different formalin fixation times. Cμ gene amplification was detected within 1 week, but faded gradually after 2-3 weeks and had disappeared at 4

weeks (Fig. 3). In human research, it has been reported that IgH gene amplification with good sensitivity is possible up to 15 days after formalin fixation [Camilleri-Broet *et al.*, 2000]. Although tissue sample size could affect the duration of DNA preservation in formalin, I suggest that PARR using FFPE samples is viable provided an appropriate fixation time is used (up to one week).

To evaluate the application of SSCP analysis for conventional PARR, 16 unfixed tissues were used as samples. In the samples, which showed a discrete band in the conventional PARR, distinct bands (two or more bands) were detected in the PARR-SSCP (Fig. 4A', lanes 1-4 and Fig. 4B', lanes 9-12). In contrast, the samples, which showed smears or obscure bands in the conventional PARR, did not show any bands in the PARR-SSCP (Fig. 4A', lanes 5-8 and Fig. 4B', lanes 13-16). In the FFPE tissues, distinct bands were also detected in the conventional PARR positive samples but no band was detected in the negative samples (Fig. 2A' and 2B'). Some samples showed more than two bands in PARR-SSCP (ex. Fig. 4B', lane 11). Theoretically, two bands should be shown in the PARR-SSCP of a monoclonal lymphoid tumor sample because each double-strand DNA molecule separates into 2 single strands [Han *et al.*, 2004]. However, in some reports there were several bands present in PARR-SSCP apart from the target bands, although the cause of this is unknown [Crisi *et al.*, 2002, Kadin *et al.*, 2001, Orba *et al.*, 2003, Signoretti *et al.*, 1999]. Although I could not elucidate the reason for this, my results show that SSCP analysis helps the determination of PARR clonality in samples showing an obscure band in conventional PARR.

To confirm the clonality results obtained in this study, sequence analyses were performed. In the IgH major positive sample (Fig. 2A and 2A', lane 2), the sequences of the 10 clones were completely identical (accession No. AB467330), confirming that the

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. 2A and 2A', lane 4), the IgH sequences of three clones were identical, but those of the remaining seven clones were different from each other (accession No. AB467331 - AB467338), suggesting that the obscure band observed in the PARR-FFPE (Fig. 2A, 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, suggesting that PARR-FFPE will provide useful information in retrospective studies of canine lymphoid neoplasia. However, because I only analyzed Cμ amplification for the 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 one week or less is appropriate for PARR-FFPE. In addition, this study 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.

CONCLUSION

Lymphomas arise in not only lymphoid tissues but also almost any tissue in the body. Diagnosis of canine lymphoma relies on the cytologic or histologic examination of the affected tissue, and is often straightforward. However, it is sometimes difficult for clinicians to biopsy according to the anatomical location of lymphoma. Furthermore, it is difficult for pathologists to diagnosis in some cases, where biopsy samples contain large numbers of reactive lymphocytes, or do not fully represent the lesion.

PARR has been developed for the diagnosis of lymphoid neoplasia [Burnett et al., 2003, Valli et al., 2006]. In PARR, the variable regions of Ig heavy chain and TCRγ genes are amplified by primers specific for the conserved regions of V and J genes, followed by size separation of the PCR products. If one or more distinct bands were observed, the results were considered to be positive. If no bands, some smears, or a ladder of faint bands were observed, the results were considered to be negative. PARR is highly sensitive and specific method for the diagnosis of lymphoid neoplasia. Furthermore, PARR can detect a small number of neoplastic lymphocytes in biopsy specimens that contains numerous reactive lymphocytes. However, PARR is a new method, whose information for the availability in clinical usage is limited. Therefore, more information of PARR as a diagnostic method for canine lymphoid neoplasia is necessary. Additionally, 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. From these backgrounds, I studied to clarify the property of PARR for canine lymphoid neoplasia.

In chapter 1 of this thesis, I evaluated the usefulness of PARR using endoscopic biopsy specimens for the diagnosis of alimentary lymphoma. Endoscopic diagnosis of

alimentary lymphoma is often difficult clinically and histopathologically. I compared PARR to pathological examination using endoscopic biopsy specimens obtained from the lesion. As the result, PARR could detect all the lymphoma cases diagnosed histopathologically, as well as latent alimentary lymphoma cases, which were diagnosed as enteritis histopathologically. Furthermore, the overall survival time of TCRγ- positive enteritis cases was significantly shorter than that of the TCRγ- negative enteritis cases. I showed that TCRγ- positive result in PARR may imply poor prognosis. These results showed that PARR using endoscopic biopsy specimens is useful for suspected alimentary lymphoma cases.

In chapter 2, I evaluated the possibility of PARR using formalin-fixed paraffin-embedded tissues for retrospective PARR studies. In some samples, control DNA amplification was successful, but not successful in the other samples. PARR-FFPE was successful in the samples, where control DNA can be amplified. The formalin-fixing durations of PARR-FFPE successful samples were shorter than those of the others. Analysis of the formalin-fixation time and DNA amplification controls suggested that long-term formalin fixation inhibits PARR, and a formalin-fixing duration of one week or less is appropriate for PARR. These results showed that PARR-FFPE is available when using tissues with an appropriate fixation time, suggesting that PARR-FFPE will provide useful information in retrospective studies of canine lymphoid neoplasia.

Furthermore, I applied single-strand conformation polymorphism analysis for improving diagnostic accuracy of PARR. In conventional PARR, decision of clonality is sometimes difficult, because the amplified DNA is separated only based on its size. SSCP analysis separates different DNA sequences based on their single-strand

secondary structure conformations. In this study, PARR-SSCP provided clearer results than conventional PARR in both unfixed and FFPE tissues. PARR-SSCP is a simple, reliable, and clear method, and could 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.

It is difficult to diagnose in some lymphoid neoplasia, including alimentary lymphoma. More sensitive and objective diagnostic examination is desirable for detecting lymphoid malignancies in dogs. PARR is highly sensitive and specific method for the diagnosis of lymphoid neoplasia, and can detect a small number of neoplastic lymphocytes in biopsy specimens. However, the information of canine PARR in clinical usage is limited, and there are some problems in order to be improved. In this thesis, I represented the usefulness of PARR in the diagnosis of alimentary lymphoma, which it is difficult to diagnose. Furthermore, I showed availabilities of PARR-FFPE and PARR-SSCP for the improvement of PARR. Although further studies were necessary, these results provide important information for clinical applications of PARR.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude and appreciation to Dr. Masaru Okuda, my supervisor, for creating the pleasant conditions necessary for the success of this work. I am greateful to Prof. Takafumi Onishi, Prof. Hisashi Inokuma, Dr. Takuya Mizuno (Laboratory of Veterinary Internal Medicine) and Dr. Kazuhiro Itamoto (Laboratory of Veterinary Surgery) for their assaistance in this study. Finally, I am highly indebted to all my friends and all members in the Laboratory of Veterinary Internal Medicine, Yamaguchi University.

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Table 1. A comparison of the TCRγ- positive and negative cases in the enteritis group

	TCRγ (+)	n[b)]	TCRγ (-)	n[b)]	*p* value
Age[a)] (years)	8.3 ± 3.7	6	6.0 ± 3.4	63	0.157
Clinical signs					
Vomiting	5	6	38	63	0.398
Diarrhea	4	6	32	63	0.675
Anorexia	3	6	25	63	0.681
Lethargy	1	6	20	63	0.659
Effusion	0	6	4	63	1.000
Weight Loss	1	4	5	37	0.483
Albumin[a)] (g/dl)	1.9 ± 0.3	5	2.5 ± 0.9	57	0.070

a) Continuous data is reported as the mean ± standard deviation.
b) The number of cases whose data was complete

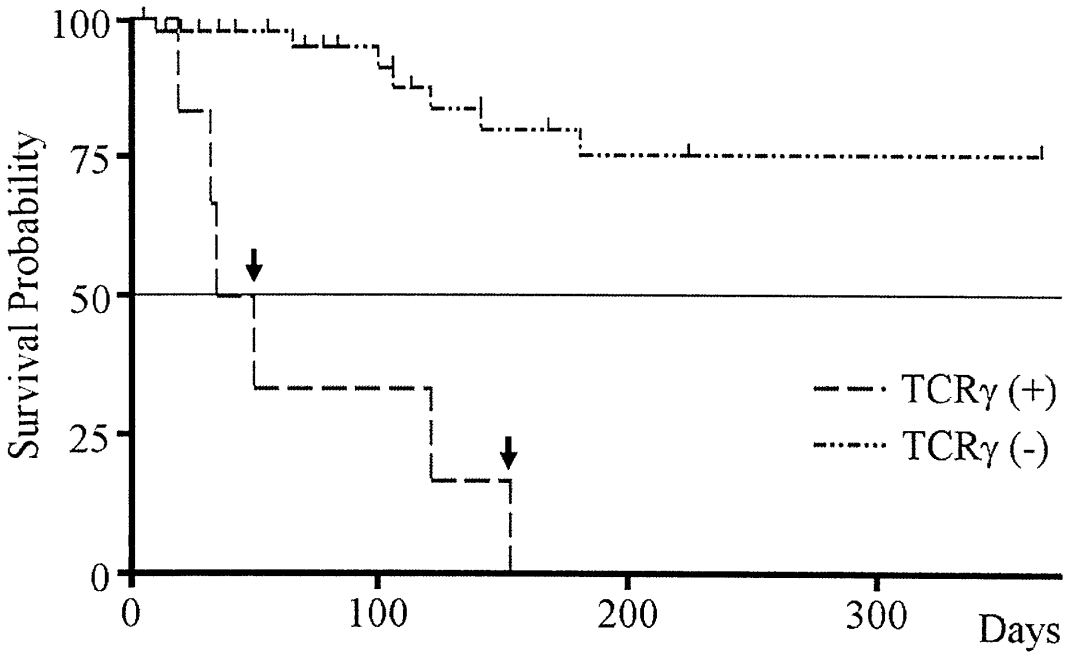

Fig. 1. The Kaplan-Meier curve for survival times of TCRγ- positive and negative cases in the enteritis group. There were six and 45 TCRγ- positive (+) and negative (-) cases, respectively. The arrows indicate the survival times of the TCRγ (+) cases diagnosed as lymphoma cytologically during laparotomy. Censored cases, which involved death by other diseases or which could not be followed up subsequently, are indicated by tick marks.

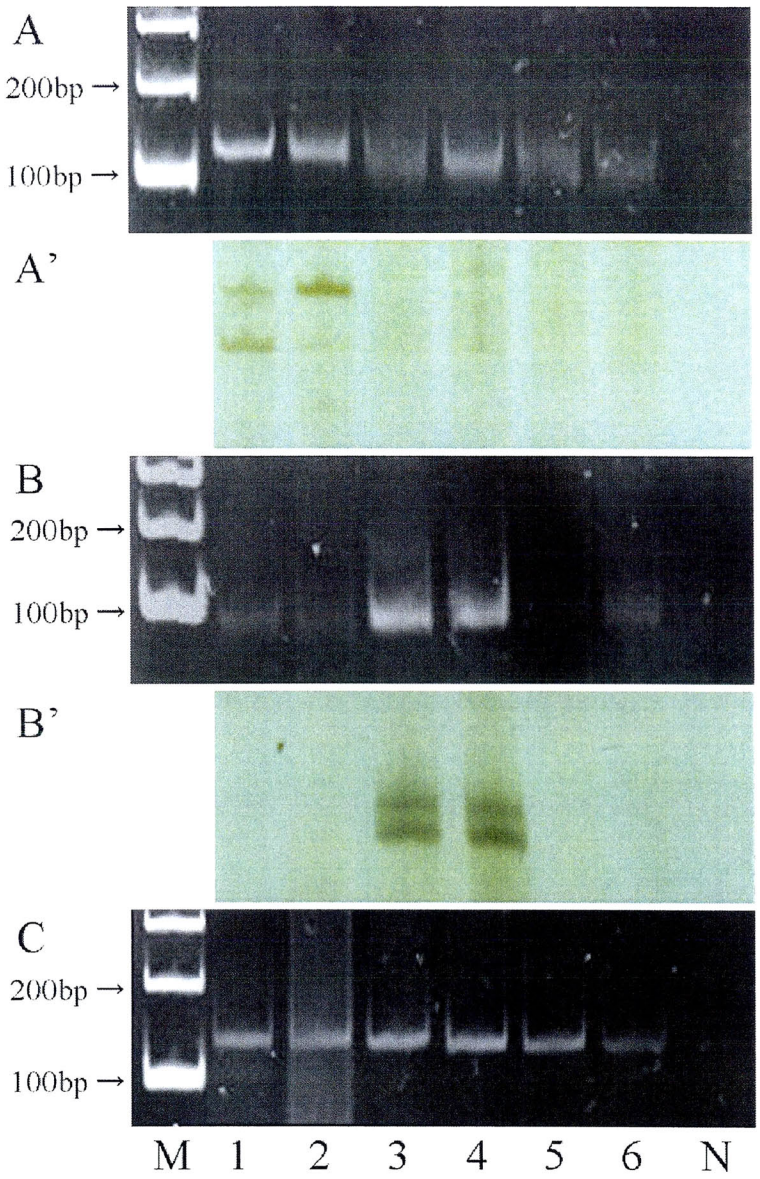

Fig. 2. Conventional PARR and PARR-SSCP for unfixed and FFPE tissues.

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).

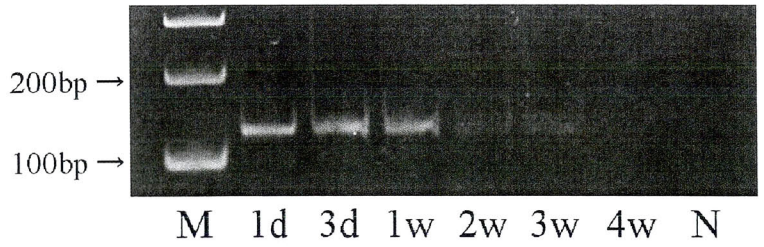

Fig. 3. Cμ gene amplification efficacy after various formalin-fixation times.

Equally sized carcinoma tissues 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).

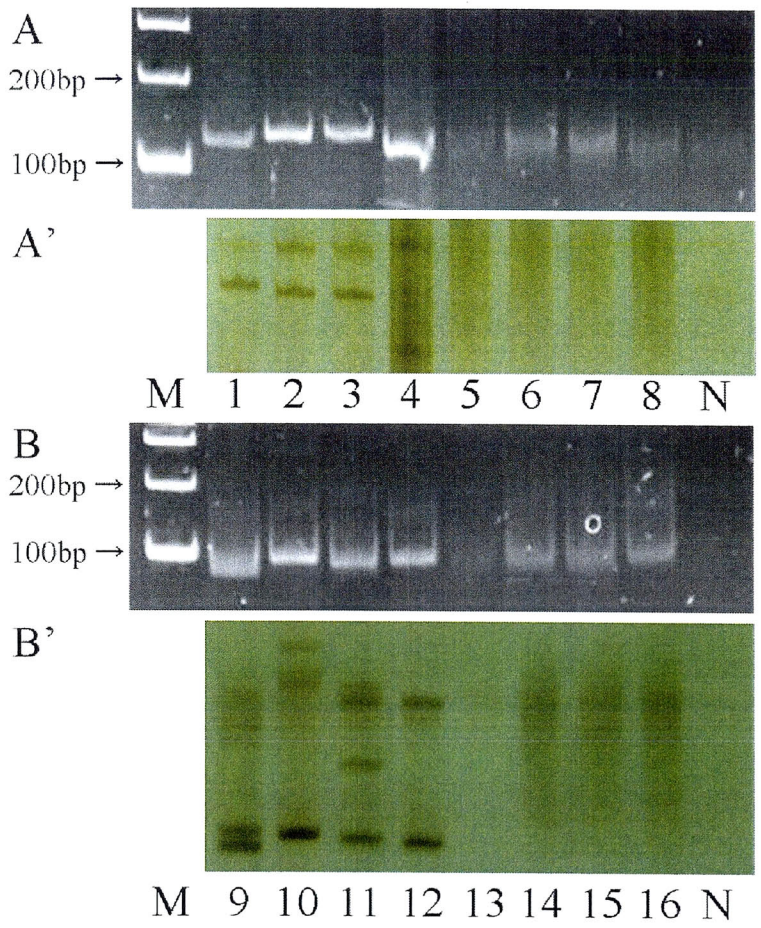

Fig. 4. A comparison of conventional PARR and PARR-SSCP in unfixed tissues.
Sixteen specimens that were obtained from clinical cases were examined with conventional PARR (A & B) and PARR-SSCP (A' & B') [Eight specimens for IgH major (A & A') and eight specimens for TCRγ (B & B')]. Lane M: 100 bp marker; Lane N: negative control (without DNA).