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Detection of Canine Lymphoma by the Amplification of Antigen Receptor Gene Rearrangements

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Abstract : We performed the PARR (PCR to detect antigen receptor rearrangements) test on DNA isolated from twelve archival canine cytological slides including nine lymphoma, two reactive lymphocytes and one sample from *Ehrlichia canis* infected dog. As a result, our PCR control gene, Cμ, was successfully amplified from all of the DNA samples. Six out of nine lymphoma samples showed a clonal rearrangement of immunoglobulin gene whereas three samples did a clonal rearrangement of T cell receptor gamma (TCRγ) gene. However, we observed no visible or clear bands from PCR conducted using our antigen receptor rearrangement primers on DNA from a reactive lymphoid cell proliferation used as a negative control. False-positive amplification in TCRγ gene was observed only in one sample from *E. canis* infection. The use of archival cytological specimens demonstrated in this study offers potential advantages for cost-effective specimen acquisition and efficient high-fidelity DNA analysis.

Key words **:** PARR (PCR to detect antigen receptor rearrangements), lymphoma, cytological slides.

Introduction

Most cases of lymphoid malignancy can be distinguished from reactive lymphoproliferations by traditional morphological analysis of the lymphocytes, alone or in conjunction with antibody staining of the cell surface and intracellular/nuclear antigens (2). However, difficulties in making a final diagnosis of lymphoid malignancy occur in a proportion of cases (5-10%), despite extensive immunophenotyping (14).

During early lymphoid development, the many different variable (V), diversity (D) and joining (J) gene segments of the Ig and TCR gene loci undergo recombination. In B cells, recombination of the V, D, and J gene segments produces the CDR3 (complementarity determining region 3) that encodes the antigen-binding portion of the immunoglobulin (Ig) heavy chain. In T cells, the unique CDR3 region encoding T-cell receptor gamma (TCRγ) is produced by recombination of the V and J regions (9,11).

PCR to detect antigen receptor rearrangements (PARR), with primers for conserved regions of the V and J genes, has been demonstrated in canine clonal lymphocyte populations. PARR relies on the amplification of tumor markers such as specific chromosome aberrations or Ig chain and T-cell receptor (TCR) rearrangements based on the assumption that all cells of a malignancy have a common clonal origin (9,5). It is so accurate and sensitive that the detection limit is 1 neoplastic lymphocyte in a population of 100 heterogeneous, non-neoplastic lymphocytes (1).

Nowadays, it is estimated that as many as 10% of clinical laboratory tests are based on the analysis of DNA, RNA, or proteins from tumor and control tissues (8). Unlike other molecular techniques, PARR does not require high molecular weight DNA for successful analysis of biological specimens. Thus, it could be ideally suitable to use a template extracted from archival clinical specimens for PARR. As such, it allows large-scale retrospective studies (7,16).

In this study, we aimed at validating the diagnosis of B- and T- cell lymphomas in dogs using PARR analysis of DNA from archival cytological slides.

Materials and Methods

Samples

A total of twelve cytological slides referred to the Chonbuk National University for the diagnosis of PARR included in this study. Selected cases were as follow: nine samples from peripheral blood or specimens from lymph node, liver and kidney collected by fine needle aspiration (FNA) from suspected cases of lymphoma based on cytological evaluation; two samples showing reactive lymphocytes in cytological examination from lymph node; one sample showing positive for *Ehrlichia canis* ELISA test (SNAP 3Dx, IDEXX Laboratories, Westbrook, USA) without lymphoid malignancy from lymph node.

Routinely stained or unstained slides were and stored at

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room temperature in an air-conditioned storage room. All samples were analyzed by PARR and three samples of reactive lymphocytes and *E. canis* infection without lymphoid malignancy were used as negative control.

DNA extraction

The DNA extraction from archival cytological slides was performed using a commercially available kit (Exgene™ Cell SV, GeneAll® Biotechnology, Korea) with some modifications. First, smears were scraped off the glass slides in a laminar flow hood with a sterile scalpel blade, and the resulting powdered material was resuspended in 200 μL of cell lysis buffer and incubated at 90ºC for 10 min. Then, 20 μL of proteinase K (20 mg/mL) was added, and the sample was incubated at 55ºC for 1 hour, and then again at 70ºC for 10 min after the addition of 200 μL of BL buffer. After the sample was cooled at room temperature, 200 μL of absolute ethanol was added, and the DNA was bound and washed using spin columns according to the manufacturer's instructions. Finally, the DNA was eluted in 50 μL of elution buffer, and the DNA concentration was measured using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Wilmington, USA).

Polymerase Chain Reaction

Amplification of immunoglobulin and T-cell receptor gamma sequences was performed using previously described primers (1). Primers used for the amplification of Ig major and minor CDR3 and TCRγ CDR3 sequences are, CB1/CB2 (5'-CAG CCT GAG AGC CGA GGA CAC- 3'/5'- TGA GGA GAC GGT GAC CAG GGT- 3'), CB1/CB3 (5'- TGA GGA CAC AAA GAG TGA GG- 3') and TCRγ1/ TCRγ2/ TCRγ3 (5'- ACC CTG AGA ATT GTG CCA GG- 3'/5'- GTT ACT ATA AAC CTG GTA AC-3'/5'- TCT GGG A/GTG TAC/T TAC TGT GCT GTC TGG- 3'), respectively. To ensure the DNA could be amplified, positive control primers were used to amplify the constant region gene of IgM (Cμ). The primers of Cμ are Sigmf1/Srμ3 (5'- TTC CCC CTC ATC ACC TGT GA- 3'/5'- GGT TGT TGA TTG CAC TGA GG- 3').

Approximately 50~100 ng of DNA was amplified with 500 nM of each primer in a 20 μL reaction volume using 1× Go Taq® Green Master Mix (Promega®, Madison, USA). The PCR assay was optimized using a DNA thermal cycler (PTC-200, MJ research, USA) with the following reaction conditions: initial denaturation for 2 min at 95ºC, followed by 35 cycles of 95ºC for 8 seconds, 60ºC for 10 sec, and 72ºC for 15 sec. No final extension time was used.

To avoid false positive results when the products were assessed with standard PAGE (polyacrylamide gel electrophoresis) techniques, a heteroduplex analysis assay was done on the DNA. This consisted of heat denaturation at 95ºC for 5 min, rapid cooling at 4ºC for 1 hour, and then PAGE. Since this process separates PCR products on the basis of junctional diversity in addition to size, it is particularly useful in the analysis of loci with restricted junctional diversity (14).

Detection of PCR products and interpretation of results

PCR products were separated on 12% native polyacrylamide gel (1.5 mm thick) by electrophoresis using 1× Tris borate EDTA buffer at 100V for 90 min. Subsequent visualization was done with a UV trans illumination system (Gel Doc 2000, Bio Rad, USA) following ethidium bromide (0.1 μg/mL in H_2O) staining for 20 min. The Cμ product is approximately 130 bp, the Ig products center around 120 bp and the TCRγ products center around 90 bp. A reaction was considered positive (clonal) if one or more dominant and discrete bands were present on the gel after electrophoresis. A reaction was considered negative (not clonal) if no band, a diffuse smear, or a ladder of faint bands was observed. In samples consisting of clonal lymphoid cells, the PCR products of rearranged Ig genes give rise to homoduplexes after denaturation and renaturation. In samples that contain polyclonal lymphoid cell populations, the single-stranded PCR fragments will mainly form heteroduplexes, which result in a background smear of slowly migrating fragments upon electrophoresis.

Results

DNA extraction was successfully performed, yielding solutions with DNA concentrations of 20 ng/μL to 1000 ng/μL. Indeed, the Cμ genes for positive control of DNA extraction were amplified in all samples except a negative control (no DNA added control).

In the cases of reactive lymphocytes, clonal rearrangements of immunoglobulin gene and TCRγ gene were not observed (i.e., amplicon was not amplified or diffuse smear bands were found). However, a clonal rearrangement of TCRγ gene showed false positive in *E. canis* infection (case 1~3 in Fig. 1).

For the six cases showing B-cell malignancies, the "Ig H major" primers, which were specific for the majority of immunoglobulin gene rearrangements were amplified in 4 cases (case 4, 5, 7 and 8 in Fig. 1) whereas the "Ig H minor" primers, which are specific for a smaller proportion of immunoglobulin rearrangements, successfully amplified DNA in all cases. In addition, three cases of T-cell lymphoma also showed TCRγ gene rearrangement. For case 11 and case 12, the Ig H minor primers were amplified and showed one discrete band with smear ladder like bands (Fig 1).

Discussion

Cytological slide smears of cells are commonly available in many laboratories as archival clinical material. Because the handling of fresh specimens is difficult, a method to use slide smears of blood or fine needle aspiration as a source would be advantageous. Indeed, some previous reports have shown the possibility of extracting amplifiable DNA and RNA from air-dried, unstained archival bone marrow slides (6), as well as from archival stained peripheral blood smears (4,18,12) and archival cytogenetic slides (10). The use of archival cytological

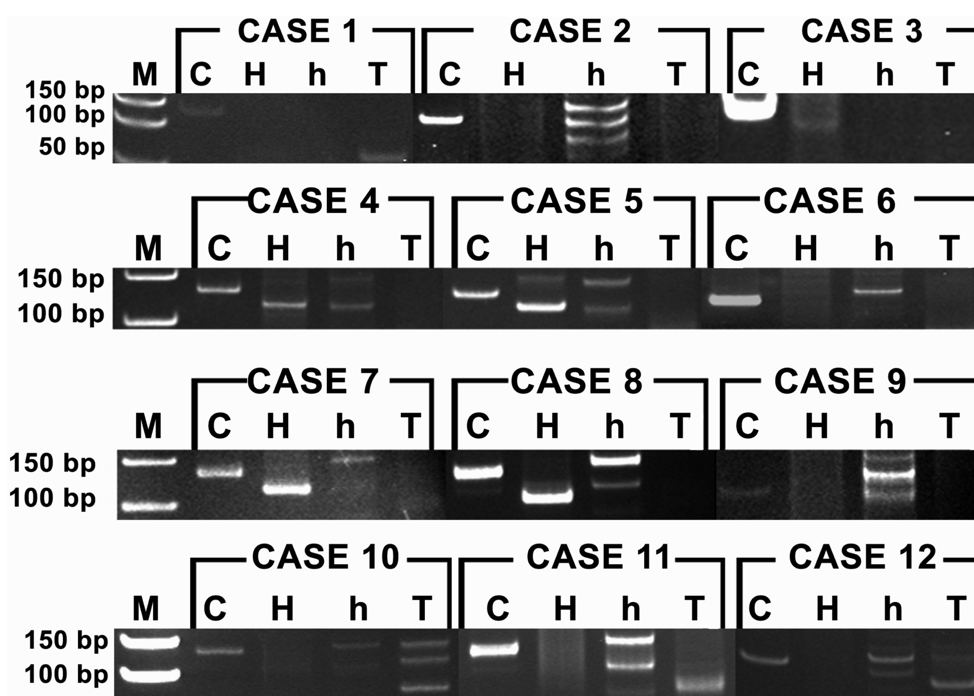

Fig 1. The results of PARR (PCR to detect antigen receptor rearrangements) from archival cytological slides. Amplicons were loaded on 12% polyacrylamide gel for electrophoresis. PCR was performed using primers for the positive DNA control, Cμ (lane C), Ig H major (lane H), Ig H minor (lane h) and TCRγ (lane T). Each lane contains PCR products from the following cases: CASE 1, *E. canis* infection; CASE 2 and 3, reactive lymphocytes; CASE 4~9, B-cell lymphoma; CASE 10~12, T-cell lymphoma.

specimens, as demonstrated in these studies, offers potential advantages for cost-effective specimen acquisition for the purpose of efficient, high-fidelity DNA analysis. Furthermore, such a technique would give the opportunity to investigate large-scale retrospective studies, as described above. Therefore the archival cytological slides are suitable source for PARR without loss of information caused by previous routine laboratory handling.

The assessment of clonal rearrangements of antigen receptor genes has also been recently introduced into veterinary medicine (15,1,3,13,17). The diagnostic method we used could improve retrospective cytological studies on archival clinical specimens and also be valuable for molecular diagnosis. The results showed that the sensitivity of the PARR for the diagnosis of the canine lymphoma was 100%, including B-cell malignancies and T-cell malignancies. In addition, clonal rearrangement of immunoglobulin and TCRγ were not observed in the cases of reactive lymphocytes. These results indicate that PARR from cytological slides can be a sensitive and reliable enough for use in veterinary clinical settings.

For the case of *E. canis* infection, however, showed clonality in TCRγ gene and this result was accordance with previous reports (1,15). Burnett *et al*. explained this phenomenon might be due to similar mechanisms to human T-cell lymphotropic virus (HTLV) or Epstein-Barr virus. *E. canis* infection should be clarified in the case showing a clonal rearrangement of TCRγ gene. Because several limitations and pitfalls might hamper the interpretation of the molecular diagnosis in the cases of false-positive results or cellular lineage and ambiguity due to pseudoclonality and oligoclonality, molecular diagnosis can be more useful when it support diagnosis in the case of: any suspect B-cell proliferations when morphology and immunophenotyping are not conclusive; all suspect T-cell proliferations; lymphoproliferations in immunodeficient patients, including post-transplant patients; evaluation of the clonal relationship between two lymphoid malignancies in one patient or discrimination between a relapse and a second malignancy; further classification of a malignancy via Ig/TCR gene rearrangement patterns or particular chromosome aberrations; staging of lymphomas (14).

In concluded, the use of archival cytological specimens demonstrated in this study offers potential advantages for cost-effective specimen acquisition and efficient high-fidelity DNA analysis.

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재배열 항원 수용체 유전자 증폭을 통한 개 림프종의 진단

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요 약 : 개의 림프종을 분자유전자학적으로 진단하고자 본 연구를 수행하였다. 이를 위하여 12개의 염색 및 고정된 도말 표본으로부터 DNA를 추출한 후, 임파육종에 특이적인 재배열된 항원 수용체 유전자를 중합효소연쇄반응으로 증폭시켰다. 그 결과 6개의 type-B 림프종에서 type-B 세포의 림프종에 특이적인 IgH (immunoglobulin H) 주유전자(major gene)와 type-B 림프종의 부유전자(minor gene)가 증폭되었다. 또한, 3개의 type-T 세포의 림프종의 경우에도 이에 특이적인 TCRγ 유전자가 증폭되었다. 한편, 에를리키아증에 이환된 표본은 TCRγ 유전자가 위양성으로 증폭되었지만, 활성화된 림프절 샘플의 경우에는 어떤 유전자의 재배열도 관찰되지 않았다. 따라서 림프종의 진단에 있어서 혈액 또는 조직 도말 표본의 DNA를 이용하여 재배열된 항원 수용체 유전자의 검출을 시도한다면, 특이적이고 객관적으로 림프종을 감별진단 할 수 있을 것이며, 나아가 회고적 분석에도 유용하게 활용될 수 있을 것으로 사료된다.

주요어 : 재배열 항원 수용체 유전자, 림프종, 중합효소연쇄반응.