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Polymerase chain reaction for antigen receptor rearrangements (PARR) for diagnosis of canine lymphoid neoplasia

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Abstract
Lymphoma is a commonly reported neoplasia in dogs. In hematological neoplasms, clonality plays a major role and the diagnostic approaches for characterization and monitoring of the lymphoproliferative disorders in humans are based on the clonality assessment. In canines, the routinely applied biochemical or histological approaches may not be potential application for early detection. A study was conducted in dogs to assess the diagnostic utility of clonality assay for lymphoid neoplasia using whole blood samples. Samples were collected from 74 suspected cases with lymphadenopathy and PARR was performed with different panel of primers and 77.02% cases were positive for clonal expansion. Among the samples that tested positive for the clonality, 96.49% of the cases were of T phenotype and 3.51% cases were of B phenotype. 14.86% of samples were positive for *E. canis* infection. The correlation of the clinical diagnosis of lymphoma with the PARR assay result was 83.33% among the tested samples.

Keywords: PARR, canine, clonality, lymphoma, B cell and T cell

1. Introduction
Lymphoma is a commonly reported hematopoietic neoplasm in dogs with a reported incidence of 13-24 per 100,000 dogs at risk and accounts for about 7% to 24% of all canine neoplasms (Kaiser, 1981; Harvey, 1990) [10, 9]. Lymphoma has been shown to be prevalent in breeds such as Labrador Retrievers, Dachshund, Pomeranian, German Shepherd, Golden Retriever and Rottweiler (Bromberek *et al*., 2016) [3]. The conventional diagnostic approach in canines under Indian conditions relies on the histological examination of the lymphoid tissue biopsy or the cytological scoring of the fine needle aspirate biopsy (FNAB).
In haematological neoplasms, clonality plays a major role and diagnostic assays that specifically detect a group of cells arising from a single clone are considered to be sensitive and specific. In addition, specificity to the neoplastic transformation of lymphocytes can be achieved only with assays that target the specific sequence of DNA. In the normal process, the mature B or T cell divides upon activation by antigenic stimulation. The antigen-binding region of the activated respective B or T cell receptor gene namely the CDR3 region of the Immunoglobulin (Ig) or T-cell receptor respectively is unique (Delves and Roitt, 2000; Blom and Spits, 2006) [6, 2]. The uniqueness and diversity arises from the recombination of V, D and J genes in B cells and V and J genes in T cells (Schatz *et al*., 1992) [3].
The clonality detection assays thus target the Ig genes in the B cells and the T cell receptor (TCR) genes in the T cells. In the case of neoplastic cells, the cells have an uncontrolled expansion and if a population of cells have the same Ig or TCR gene, then the case is more likely to be neoplastic than reactive (Swerdlow, 2003) [14]. Thus the appearance of single-sized dominant PCR amplicon in the PCR based approaches confirms the clonality and hence the assay is termed as PCR for antigen receptor rearrangement (PARR). The PARR method enables detection of even one neoplastic cell in 100 cells in non-lymphoid tissue while less sensitive in lymphoid tissue and thus helpful in identifying neoplastic lymphocytes earlier than cytology (Avery, 2009). The primer pair reported by Burnett *et al.*, in 2003 [4] is commonly employed to detect the clonal rearrangement of the antigen receptor genes and offers 94% specificity and 75% sensitivity. However, Keller and Moore (2012) [11] reported drawbacks in the primer design with earlier published reports and insisted the need to cover all rearranged genes to significantly decrease false negative results. Hence, screening of samples with panels of different primers that cover a wide variety of sequence divergence will improve the

sensitivity of the PARR assay. In this study, we tested whole blood samples from 74 canine patients and used a panel of primer pairs to detect the rearrangement in the Ig and TCR and correlated a cohort of samples to cytology as well as to the positivity to *E. canis*

2. Materials and Methods

2.1 Selection of animals & Collection of samples

The animals that were brought to the Department of Clinics at Madras Veterinary College, Chennai with clinical observation of lymphadenopathy and splenomegaly were mainly suspected for lymphoma and ultrasound imaging was performed in these cases to confirm the internal lymph node involvement. Such animals were selected for the study and whole blood samples were collected for blood biochemistry and PARR assay. All the samples were collected with the consent of the pet-parent for the purpose of diagnosis and prognosis. The study included 74 canine samples collected between 2019 and 2020.

2.2 DNA extraction from whole blood

Genomic DNA was extracted from the whole blood samples using DNAzol method following manufacturer's instructions. The RBCs were lysed with RBC lysis buffer and the remaining WBC cell pellet was lysed using DNAzol. The lysate was extracted once with Phenol: Chloroform: Isoamyl Alcohol mixture (25:24:1) and the collected aqueous phase was extracted with Chloroform: Isoamyl Alcohol (24:1). From the aqueous phase collected after this step, the DNA was precipitated with Isopropanol, washed with 70% Ethanol and the final DNA pellet was resuspended in TE buffer (10 mM Tris and 0.1 mM EDTA). The quality and quantity of the DNA samples were assessed using Nanodrop.

2.3 PARR assay to detect clonality

All the samples were screened in duplicates and the results were considered as positive only when the duplicates resulted in the specific size amplicon confirming the clonal expansion.

2.3.1 PARR with primary panel of primers

The initial assay was optimized for the primers reported by Burnett *et al*. (2003) [4]. The primer sets targeted the amplification of the constant region of IgM (i.e., the Cμ as positive control), primer sets for the immunoglobulin rearrangement to detect the B-cell clonal expansion (i.e., the IgH major and IgH minor) and one primer set for the TCRγ. The details pertaining to the primer sequences are provided in the Table 1. For the PCR reaction, the reaction mixture was initially denatured at 95 °C for 5 minutes followed by 30 cycles of 94 °C for 20 seconds, 60 °C for 20 seconds and 72 °C for 45 seconds and final extension at 72 °C for 1 minute. The PCR amplicons were electrophoresed in a 4% Tris acetate EDTA agarose gel and the results were documented.

2.3.2 PARR additional primer panel for B cell proliferation

The PCR for the B cell proliferations included the primers with slight modifications as reported by Gentilini *et al*. (2009) [8] and Tamura *et al*. (2006) [15] and details on the primer sequences are given in the Table 2. The PCR reaction included initial denaturation at 95 °C for 5 minutes followed by 30 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 30 seconds and a final extension of 72 °C was for 10 minutes. The PCR amplicons were agarose electrophoresed as described above.

2.3.3 PARR additional primer panel for T cell proliferation and suspected *Ehrlichia canis* infection

Clonal TCRγ rearrangements have been identified and reported in dogs with Ehrlichiosis (Vernau and Moore, 1999; Burnett *et al*., 2003) [4, 17] and such T cells could be transformed T cells that have not evolved to overt neoplasia as reported in HTLV infection in humans. Hence, to rule out the possibility, the samples were also screened for *E. canis* infection with species-specific primers and also with additional set of primers TCRγ N1 and TCRγ N2 for the T cell proliferation (Table 3). The PCR amplicons were agarose electrophoresed as described above.

Table 1: Details of the primer sequences and their combination applied for the detection and differentiation of T and B cell Clonal expansion

S. No.	Gene Target	Primer name	Primer sequence (5' – 3') *	Amplicon size
1.	Cμ	Sigmf1	TTCCCCCTCATCACCTGTGA	130 bp
		Srμ3	GGTTGTTGATTGCACTGAGG	
2.	IgH Major	CB1	CAGCCTGAGAGCCGAGGACAC	120 bp
		CB2	TGAGGAGACGGTGACCAGGGT	
3.	IgH Minor	CB1	CAGCCTGAGAGCCGAGGACAC	120 bp
		CB3	TGAGGACACAAAGAGTGAGG	
4.	TCRγ	TCRγ1	ACCCTGAGAATTGTGCCAGG	90 bp
		TCRγ2	GTTACTATAAACCTGGTAAC	
		TCRγ3	TCTGGGA/GTGTAC/TTACTGTGCTGTCTGG	
* Primer sequences as reported in Burnett *et al*. (2003) [4]				

Table 2: Details of the additional primer sequences and their combination targeting the B-cell receptor (BCR)

S. No.	Primer name	Primer sequence (5' – 3') **	Amplicon size
1.	TamuraF	ACACGGCCVTGTATTACTGT	79 – 90 bp
	CB2	TGAGGAGACGGTGACCAGGGT	
2.	CB1	CAGCCTGAGAGCCGAGGACAC	85 – 145 bp
	CB3(a)	TGAGGACACGAAGAGTGAGG	
3.	3'FWR1	GCCTCTGGATTCACCTTCAG	262 – 315 bp
	CB2	TGAGGAGACGGTGACCAGGGT	
4.	5'FWR1	GAGGTGCAGCTGGTGGAGTCT	309 – 378 bp
	CB2	TGAGGAGACGGTGACCAGGGT	
5.	3'FWR1	GCCTCTGGATTCACCTTCAG	262 – 315 bp
	CB3(a)	TGAGGACACGAAGAGTGAGG	
** Primer sequences as reported in Burnett *et al*. (2003) [4], Tamura *et al*. (2006) [15], Gentilini *et al*. (2009) [8] and Waugh *et al*. (2016) [16] and used in the combination as reported above			

Table 3: Details of the additional primer sequences targeting the T-cell receptor (TCR) and Ehrlichiosis

S. No.	Gene Target	Primer name	Primer sequence (5' – 3') [$$]	Amplicon size
1.	TCRγ New	TCRγ N1	GGCGTGTACTACTGCGCTGCC	55 – 82 bp
		TCRγ N2	TGTGCCAGGACCAADYACTTT	
2.	*E. canis*	Fwd	CAATTATTTATAGCCTCTGGCTATAGGA	409 bp
		Rev	GAGTTTGCCGGGACTTCTTCT	

[$$] Primer sequences as reported in Waugh *et al*. (2016) [16], Nazari *et al*. (2013) [12]

3. Results and Discussion

The study included a total of 74 blood samples and the DNA was extracted from these samples as described in the materials and methods. The extraction method resulted in an average DNA yield of 1121.32 ng/µl from the samples with a 260/280 ratio of 1.85 ± 0.06 indicating a good quality DNA for use in the PCR assays. The positive control for each of the DNA samples tested by the PARR assay included the use of Cµ primers along with a no template control (NTC).

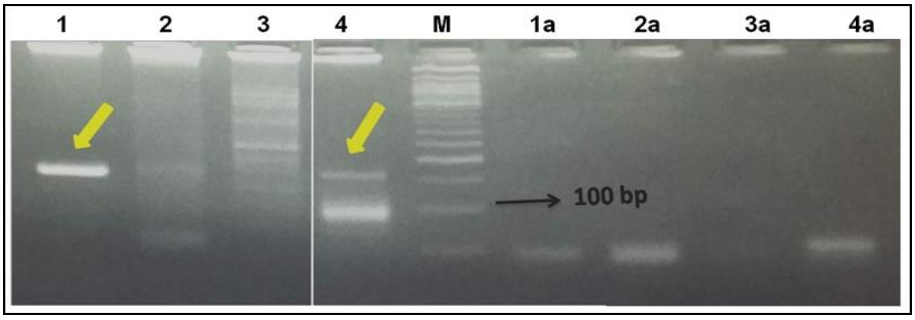

Fig 1: PARR assay performed with 100ng of PBMC DNA. Lane 1 - Cµ (Positive), Lane 2 – IgH Major (Negative), Lane 3 – IgH Minor (Negative), Lane 4 – TCRγ (Positive – Biclonal), M – 50bp ladder, Lanes 1a to 4a - NTC for Cµ, IgH Major, IgH Minor and TCRγ respectively.

Consistent amplification of a 130 bp product with the primer for the Cµ indicated the availability of sufficient DNA for the PARR analysis. PCR with the PARR primary panel of primers resulted in an amplicon size of 120 bp for the IgH major and minor and a 90 bp amplicon for the TCRγ in samples confirmed to be positive for the clonal expansion of B and T-cell proliferation respectively (Fig 1). For the PARR additional primer panel for B cell proliferations, the PCR resulted in amplicon sizes of 79-90 bp, 85-145 bp, 262-315 bp, 309-378 bp and 262-315 bp for the primers sets 1, 2, 3, 4 and 5 respectively (Fig 2). The PARR additional primer panel for T cell proliferation resulted in an amplicon of 55-82 bp (Fig 3A) and a 409 bp amplicon confirmed the sample to be positive for *Ehrlichia canis* infection (Fig 3B).

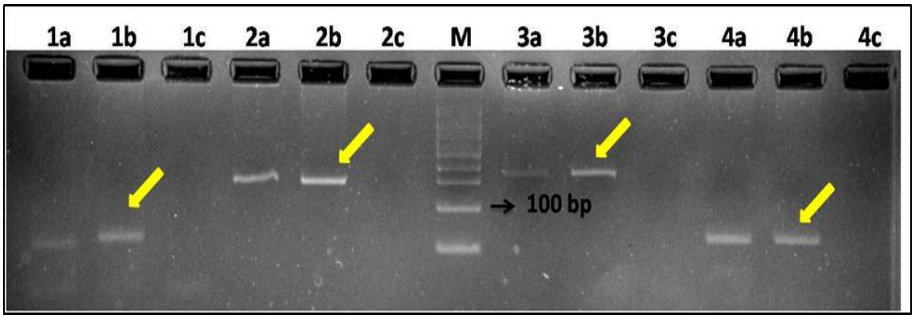

Fig 2: PARR additional primer panel for B cell proliferations. Lane 1 – IgH Major (CB1 & CB2) – 120 bp, Lane 2 – 3'FWR1 & CB2 (Set 3) – 262-315 bp, Lane 3 – 5'FWR1 & CB2 (Set 4) – 309-378 bp, Lane 4 – Cµ (Sigmf1 & Srµ3) – 130 bp, M – 100 bp ladder. a and b indicate samples and c indicate NTC.

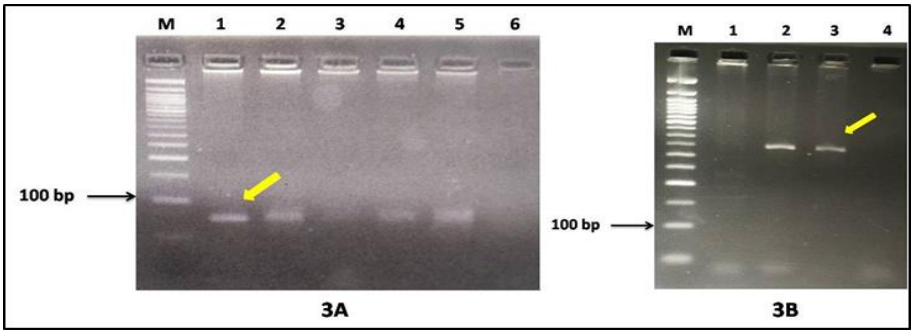

Fig 3A: PARR additional primer panel for T cell proliferation. The amplified product size is 55-82bp. Lane M is 50 bp ladder. Lanes 1, 2, 3, 4, 5 – Samples. Lane 6 – NTC.

Fig 3B: PARR additional primer panel for suspected Ehrlichia canis infection. The amplified product size is 409 bp. Lane M is 50 bp ladder. Lanes 1, 2, 3 – Samples. Lane 4 – NTC.

Out of 74 animals under study, 55 samples (74.32%) showed positivity for TCR clonal expansion with 14.86% of Ehrlichiosis and 2 samples (2.7%) showed positivity for BCR clonal expansion. In the PCR, single PCR amplicons were detected for BCR; while in most of the cases two amplicons were observed in samples positive for TCR clonal expansion (indicating biclonality). No bands or a ladder pattern observed in the samples indicated a negative result for the clonal expansion. Generally there is more prevalence of B cell lymphoma but the results in our study revealed only few of the samples to be positive for BCR. To confirm the true negativity for the clonal expansion of the BCR, we used 5 different additional primer panels to confirm the results. The positivity for lymphoma was high in the Labrador breed among the screened population and mostly in the stage III or IV.

As per earlier reports, the determination of non-lymphoma using the primer sets were CB1 and CB2 – 94%, 3'FWR1 and CB2 – 91%, 5'FWR1 and CB2 – 88%, TCRγ N1 and TCRγ N2 – 85%. The determination of B cell phenotype for the primer sets were CB1 and CB2 – 69%, 3'FWR1 and CB2 – 61%, 5'FWR1 and CB2 – 66% and for the T cell phenotype TCRγ N1 and TCRγ N2 – 67% (Ehrhart, *et al*., 2019) [7].

Cytology of a fine needle aspirate from a neoplastic lymph node is often used for the screening of lymphoma. But it generally works well with high grade lymphoma and for the low grade cases it remains insufficient. The fine needle aspirates have been used for the PARR assay by most of the earlier published reports. We have tried to optimize PARR with whole blood samples in this study. Since the animals in the study were mostly in the stage III and IV category of lymphoma, the clonal expansion was detectable in the blood samples. Out of the samples tested, only one sample tested positive with FNA and not with the whole blood which indicated initial stage of lymphoma and the absence of circulating neoplastic cell population. Among the samples positive for the clonal expansion of TCR, 11 samples resulted in the amplification of the 409 bp with the *E. canis* primer pair. In such situations, the clinician applied the specific therapeutic intervention for Ehrlichiosis and the pets showed a recovery in clinical symptoms including the observed lymphadenopathy.

To confirm the reliability of the PARR assay in detecting the clonal expansion for BCR/ TCR, a cohort of samples were also assessed by immuno-phenotyping and flow cytometry. A very good correlation was observed between the immuno-phenotyping and PARR assay indicating the reliability of the peripheral blood samples in detecting lymphoma when the animals were presented in the stage III and IV. This is also because of the increased sensitivity of the PARR assay in detecting the transformed cells when applied to non-lymphoid cells.

4. Conclusion

This study concludes that the correlation between the clinical diagnosis of lymphoma and the PARR assay result was around 83.33% among the tested samples. This study gives an insight on to the usage of blood samples for preliminary screening of lymphoma by clonality assay. Also the study concludes that there is a need for diagnosis of parasitic infections like Ehrlichiosis before prescribing the treatment regimen for canines with T cell lymphoma.

5. Acknowledgment

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