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Research Article

Diagnosis of lymphoma, chronic myeloid leukemia and mast cell tumor in dogs using polymerase chain reaction

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Abstract

In pet cancer diagnostics, there is a dire need for molecular diagnostic tools that give results in few hours rather than days. Hematopoietic cancers, particularly lymphoma, the most common type of hematopoietic cancer, have been reported in dogs. Diagnosis of blood malignancies is difficult based on histological and cytological examination. In such a situation, advanced molecular techniques may be helpful to confirm a diagnosis. It is very much essential to diagnose blood malignancies. In the present study, 123 blood samples from dogs were screened to diagnose the lymphoma using polymerase chain reaction for antigen receptor rearrangement and 10 blood samples were screened to diagnose chronic myeloid leukemia using a two-step nested polymerase chain reaction for detection of the *bcr-abl* fusion. Twenty one tissue samples from abnormal growth on the body surface of dogs were screened to diagnose mast cell tumor using polymerase chain reaction for detection of mutation at juxtamembrane domain of *c-kit* gene. Two out of 123 dogs were found to be positive for T-cell lymphoma, 3 dogs were positive for chronic myeloid leukemia out of 10 samples and 1 tissue sample was found to be positive for mast cell tumor out of 21 suspected samples. Thus, regular use of molecular diagnostics in veterinary practice for speedy and early diagnosis of tumors, particularly insidious neoplastic conditions, as well as in taking therapeutic decisions is very crucial.

Keywords: Chronic myeloid leukemia, Dog, Lymphoma, Mast cell tumor, Molecular diagnostics

Highlights

- Molecular diagnostics for rapid and accurate diagnosis of cancers.
- Two cases of T-cell lymphoma were diagnosed using PARR assay.
- Mutation in *c-kit* gene was detected in one case of canine mast cell tumor using PCR.
- Bcr-Abl chromosomal abnormality was detected in three cases of chronic myeloid leukaemia using two step nested PCR first time in dogs.

INTRODUCTION

Dogs are affected by tumors more often than other livestock species, and lymphoma is amongst the most common hematopoietic cancers (Carter and Valli, 1988). Lymphoma usually originates from lymphoid tissues like lymph nodes, spleen, and bone marrow. It accounts for approximately 7-24% of all canine neoplasia and 84% of all canine hematopoietic malignancies (Vail and Vithrow, 2007). Mast cell tumor (MCT) is another common type of tumor in dogs involving the skin. It is present in 7-21% of cases of cutaneous tumors in dogs (Ma *et al.*, 1999). Besides the skin, other parts of the body, such as the spleen, liver, gastrointestinal tract, and bone marrow, may also be affected. Mast cell tumor in dogs is caused by a mutation in the *c-kit* gene at the juxtamembrane domain (Cameron *et al.*, 2004). Chronic myeloid leukemia (CML) has also been reported in dogs and it starts in certain blood-forming cells of the bone marrow. Bcr-Abl chromosomal abnormality was reported in dogs affected with CML (Breen and Modiano, 2008).

We have X-ray, ultrasonography, and CT scan as diagnostic methods for clinical examination of tumors with limited application, i.e. they can tell us only about the presence and absence of abnormal growth. Positron emission tomography (PET), however, is a more

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advanced technique that can be employed for cancer diagnosis and staging of cancer, and in a few cases, may act as a therapeutic. Cancer could be confirmed upon cytology/histopathology examination; albeit, these are inadequate to identify the type of cells involved in tumor development particularly cancer of high grade and blood origin owing to the absence of a striking histopathological feature. Moreover, these tools are time consuming, need sophisticated machines, need appropriate samples in good condition, and interpretation of results require highly skilled and technical person. However, nucleic acids based molecular tumor diagnostic tools that give results in few hours with high accuracy, sensitivity and specificity. Moreover, they do not need highly skilled and technical person to perform and interpret the results of molecular diagnostic tools. The present study aimed to diagnose the three important neoplastic conditions, i.e. lymphoma, CML and MCT in dogs using nucleic acid based assays.

MATERIALS AND METHODS

Target population: Dog with a history of an ailment of unknown origin or an abnormal growth on their body were included in this study irrespective age, sex and breed. Samples included in this study were from Teaching Veterinary Clinical Complex of College of Veterinary and Animal Sciences, Palampur, India and various veterinary hospitals and polyclinics within the state of Himachal Pradesh, India.

Sample collection: A total of 123 blood samples in di-potassium ethylenediaminetetraacetic acid (K_2EDTA) vials and 21 tumor tissue samples in absolute alcohol were collected. DNA was extracted from blood and tissue samples using phenol-chloroform-isoamyl alcohol (PCI) method as described by Birren *et al.* (1997). Ten blood samples without coagulant were used to extract the total RNA using QIAamp® RNA Blood Mini Kit (Qiagen, US) as per kit's protocol.

Polymerase chain reaction: Polymerase chain reaction for antigen receptor rearrangement (PARR) assay for lymphoma was performed to diagnose the lymphoma in dogs. This technique was used to detect the monoclonality among B-cell and T-cell in lymphomas. The reaction conditions to carry out PCR and primer sequences were as per Burnett *et al.* (2003) (Table 1). The PCR reaction was carried out in a reaction volume of 25 µL in GeneAmp PCR System

Table 1. Primers used in the present study

Primer set	Type of product	Primer	Sequence (5′-3′)	Product size (bp)
1	Cµ	Sr3	TTCCCCCTCATCACCTGTGA	130
		Sigmf1	GGTTGTTGATTGCACTGAGG	
2	IgH major	CB1	CAGCCTGAGAGCCGAGGACAC	120
		CB2	TGAGGAGACGGTGACCAGGGT	
3	IgH minor	CB1	CAGCCTGAGAGCCGAGGACAC	120
		CB3	TGAGGACACAAAGAGTGAGG	
4	TCRγ	TCRγ 1	ACCCTGAGAATTGTGCCAGG	90
		TCRγ 2	GTTACTATAAACCTGGTAAC	
		TCRγ 3	TCTGGGA/GTGTAC/TTACTGTGCTGTCTGG	
5	*c-kit* gene	P1 (F)	CCATGTATGAAGTACAGTGGAAG	191
		P2 (R)	GTTCCCTAAAGTCATTGTTACACG	
6	First round Nested PCR	CMLNA	TGGAGCTGCAGATGCTGACCAACTCG	
		CMLNB	ATCTCCACTGGCCACAAAATCATACA	
7	Second round Nested PCR	CMLNH	GATCTCCTCTGACTATGAGCGTCCA	305
		CMLNI	TTCACCTTTAGTTATGCTTAGAGTG	
8	ß2µ	ß2M (F)	TCCTCATCCTCCTCGCT	85
		ß2M (R)	TTCTCTGCTGGGTGTCG	

9700 (Applied Biosystems, USA) thermal cycler. Approximately 40 ng DNA was amplified with 0.2 µM of each primer in 25 µL reaction. For preparation of PCR reaction GoTaq® Flexi DNA polymerase (Promega) kit was used. PCR reaction was initially denatured at 95ºC for 5 min followed by 40 cycles of 30 sec at 94ºC, 30 sec at 58ºC and 30 sec at 72ºC. A final extension step of 3 min at 72ºC was provided before analyzing the samples using electrophoresis. 5 µL of PCR amplicons were evaluated on 10% native PAGE using 1X TBE buffer, pH 8. PAGE was performed by using a mini vertical electrophoresis unit (Tarsons, India) as per instructions given in the user manual. Native PAGE consists of only resolving gel (no stacking gel as in SDS-PAGE). Electrophoresis was done at constant voltage of 100V for approximately 2 hr. Gel was stained with ethidium bromide solution for 15 min. After staining, gel was visualized and photographed (Alpha Digidoc Gel documentation system, USA).

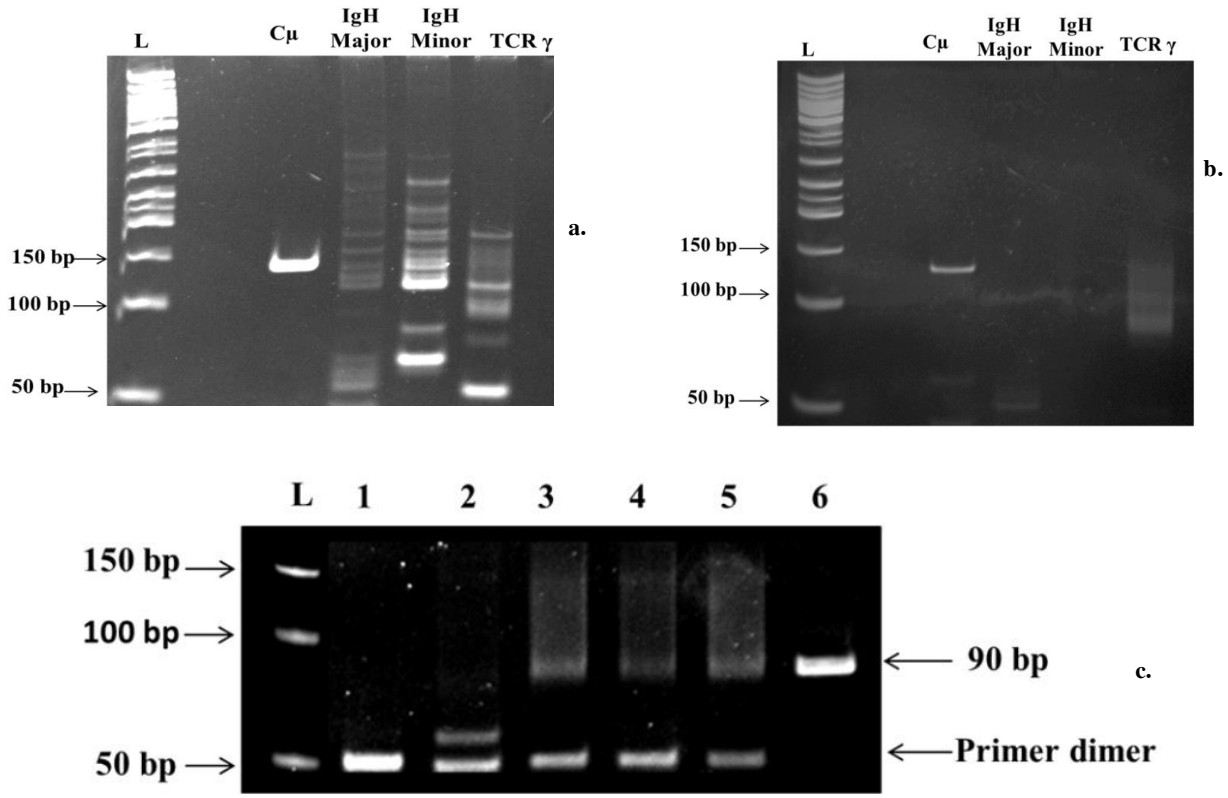
c-kit gene mutation in MCT was diagnosed as per method described by Cameron *et al.* (2004). Primers used in PCR and product size of amplified gene is given in Table 1. For preparation of PCR reaction GoTaq® Flexi DNA polymerase (Promega) kit was used. PCR reaction was carried out in a reaction volume of 25 µL in GeneAmp PCR System 9700 (Applied Biosystems, USA) thermal cycler. Approximately 40 ng DNA was amplified with 0.2 µM of each primer in 25 µL reaction. PCR amplification profile was 40 cycles of 1 min at 94ºC, 1 min at 59ºC and 1 min at 72ºC. A final extension period of 5 min at 72ºC was provided. PCR product was evaluated in 4% agarose gel (HiMedia, Mumbai). Electrophoresis was done at constant voltage of 100V for 40 min. After electrophoresis, gel was stained with ethidium bromide solution for 15 min. Stained gel was visualized under UV light in Alpha Digidoc Gel documentation system, USA.

A two-step nested RT-PCR was used performed for the diagnosis of CML in dogs. Firstly, RNA was isolated from whole blood using QIAamp® RNA Blood Mini Kit (Qiagen, US) as per kit's protocol. cDNA was synthesized using the Verso cDNA synthesis kit (Thermo Scientific, USA). PCR reaction and primers used for RT-PCR were as per Radich *et al.* (2001) (Table 1). *ß2µ* gene was taken as a positive control (Selvarajah *et al.*, 2017). First round of RT-PCR was carried out in 25 µL reaction volume. PCR amplification profile was 40 cycles of 30 sec at 94ºC, 1 min at 55ºC and 1 min at 72ºC. A final extension period of 7 min at 72ºC was carried out. PCR product of first round was used as a template for second round PCR. PCR reaction for second round was also carried out in 25 µL reaction volume. PCR reaction was initially heated at 95ºC for 30 sec followed by 40 cycles of 30 sec at 94ºC, 1 min at 55ºC and 1 min at 72ºC. A final extension period of 7 min at 72ºC was provided. The second-round PCR product was analyzed using a 2% agarose gel electrophoresis. After electrophoresis, gel was stained with ethidium bromide solution for 15 min. Stained gel was visualized under UV light in Alpha Digidoc Gel documentation system, USA.

For histopathology examination, tumor tissues were immediately fixed into 10% neutral buffer formalin after collection. A minimum time period of 72 hr were given for fixation. Formalin fixed tissues were washed under running water before undergoing for tissue processing. The paraffin wax blocks were prepared using L-molds. These paraffin blocks were stored at room temperature till tissue sectioning. Before tissue sectioning, paraffin blocks were kept inside cryo console (Histo-Line Laboratories, USA) for better tissue sectioning. Tissue sectioning was done using a microtome (Thermo Scientific, USA). Ready-to-use haematoxylin (Mayer's) and eosin stains procured from Himedia were used in the staining protocol (Fischer *et al.*, 2008). After that, slides were mounted using DPX and kept overnight at room temperature for drying. These slides were examined under a light microscope at different magnifications for histopathological examination.

RESULTS

In the present study, a total of 123 blood samples were screened by PARR assay to detect lymphoma. In PARR assay, either multiple bands in the form of a ladder or smear formation or no band indicate the absence of lymphoma (Fig. 1a and Fig. 1b). Out of 123 blood samples, monoclonality was detected in two blood samples. In both the blood samples, clonality was detected in the T-cell receptor as depicted in Fig. 1c. Out of 10 blood samples, three were found positive for Bcr-Abl fusion (Fig. 2b) in two step nested PCR indicated by presence of amplicon of 305 bp size confirming chronic myeloid leukemia. Out of 21 tumor



a. L: 50 bp DNA ladder; Cμ: Positive control for DNA (130bp); IgH major and IgHminor: No band formation; TCRã: Smear formation. b. L: 50 bp DNA ladder; Cμ: Positive control for DNA (130 bp); IgH major, IgH minor and TCRã: Multiple bands (ladder) formation. c. L: 50 bp DNA ladder; Lane 1: Blank; Lanes 2, 3, 4, 5: Negative for T-cell lymphoma; Lane 6: Positive for T-cell lymphoma.

Fig. 1. PARR assay on 10% native PAGE

tissue samples, only one tumor tissue sample was found positive for mast cell tumor in PCR (Fig. 2a) indicated by presence of a sharp band of 191 bp size without internal tandem duplications as well as in histopathological examination (Fig. 2c).

DISCUSSION

In the present study, PARR technique was used to detect the monoclonality among B-cell and T-cell in lymphomas. Immunoglobulin and T-cell receptor's CDR3 region genes encode the antigen-binding region. CDR3 region in B cells and T-cells produced by recombination of V, D, and J genes segments. In healthy dogs, there are always multiple populations of B-cells and T-cells, and each B-cell population and T-cell population have a unique CDR3 region in their receptors. As a result of that, either multiple bands in the form of a ladder or smear formation or no banding, the PCR of healthy dogs is generated (Burnett *et al.*, 2003). In the case of lymphomas, lymphocytic leukemia, and myeloma, since there is a clonal expansion of particular lymphocytes and these populations of lymphocytes contain DNA regions in their CDR3s which are similar in length as well in sequence and as a result monoclonal, biclonal or oligoclonal amplicons are produced, respectively. In the current study, out of 123 blood samples, monoclonality was detected in two blood samples. In both the blood samples, clonality was detected in the T-cell receptor. These results were similar to those documented by Burnett *et al.* (2003) and Waugh *et al.* (2016). PARR assay could, therefore, be an important diagnostic method in cases where diagnosis based on imaging or clinical observation is not possible or doubtful. In this study, six cases were suspected as lymphoma upon histopathological observations; however, after employing PARR assay, only two cases were confirmed as T-cell lymphoma (Fig. 1c). These findings emphasize the need for a combination of molecular methods in addition to histopathology/ cytology examination for confirmation of cancers of lymphoid origin as has also been suggested previously

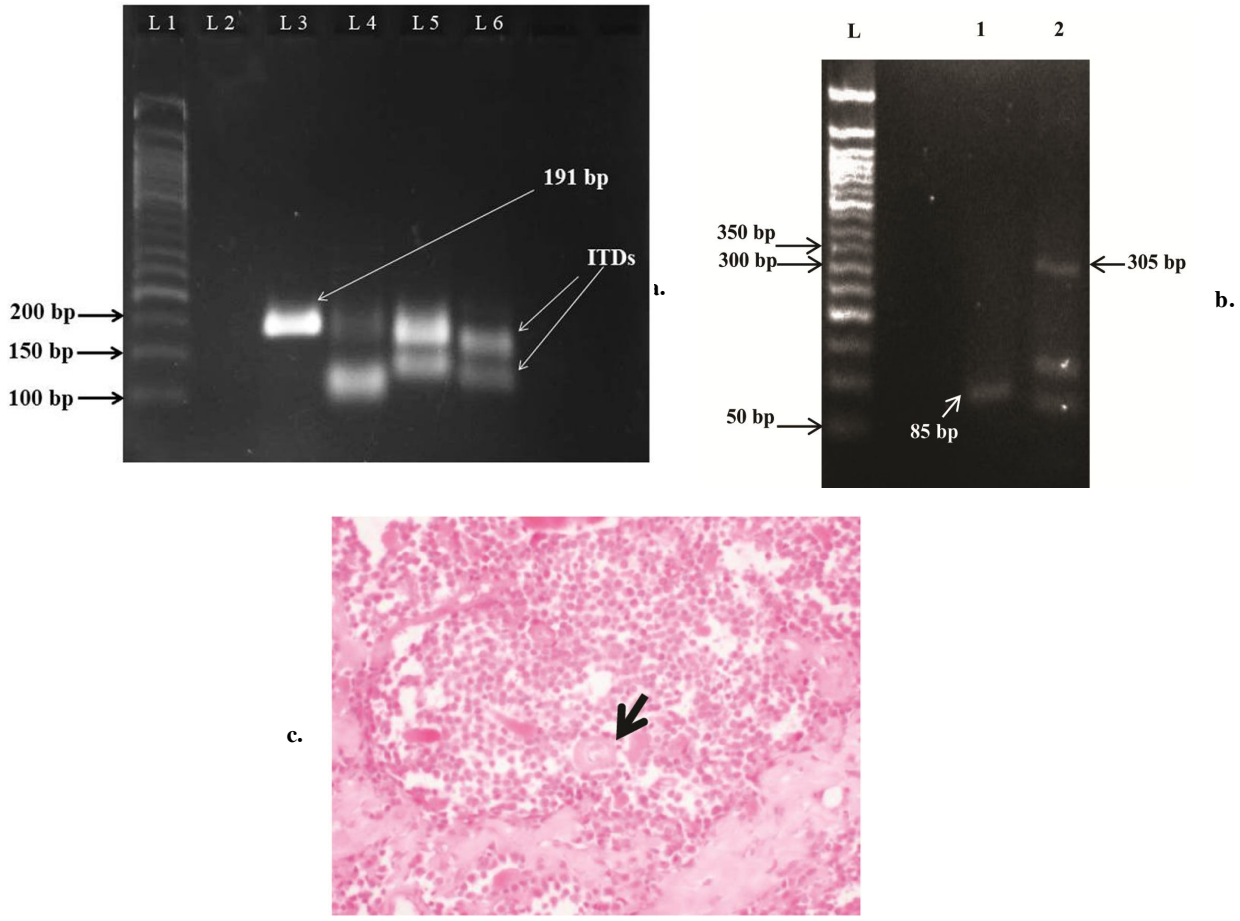


Fig. 2a. Agarose gel electrophoresis (2%) for *bcr-abl* fusion PCR amplicons - L: 50 bp DNA ladder; Lane 1: Positive internal control (β2μ); Lane 2: Positive for *bcr-abl*fusion gene (305bp)
Fig. 2b. Agarose gel electrophoresis (4%) of mast cell tumor PCR amplicons– L 1: 100 bp DNA ladder; L 2: Negative control, L 3: MCT positive sample, and L 4 to L 6 : MCT negative samples with ITDs
Fig. 2c. H & E stained tumor tissue section (under microscope 40X) - showing the characteristics of mast cell tumor (MCT) in which vein (indicated by arrow) was surrounded by a compact mass of mast cell

by Cozzolino *et al.* (2016). The PARR assay has been reported to have good sensitivity, with the detection of 98% of cases of lymphoma in dogs (Gentilini *et al.*, 2009).

The *c-kit* gene forms a transmembrane tyrosine kinase receptor that binds the ligand stem cell factor (SCF) known as mast cell growth factor (Galli *et al.*, 1994; Cardoso *et al.*, 2017). Binding of SCF helps in the development of mast cells from hematopoietic precursors and along with different cytokines, it stimulates the development and proliferation of different bone marrow progenitor cells (Nocka *et al.*, 1990). Mutations in the *c-kit* gene leading to constant activation of Kit in the absence of ligand binding have been identified in malignant mast cell lines of dog, rat, and mice (Tsujimura *et al.*, 1996; Chen *et al.*, 2016). Out of 21 tissue samples screened, one was found positive for mast cell tumors. A sharp band of 191 bp size without internal tandem duplications (ITDs) was evident on 4% agarose gel. These results were similar to Cameron *et al.* (2004). Frequent ITDs of exon 11 and 12 of the *c-kit* gene have been documented in healthy dogs but are absent in MCT (Downing *et al.*, 2002). ITDs have a size range of 3-79 bp (Zemke *et al.*, 2002). This positive sample was also found positive for MCT using histopathological examination.

In the present study, three out of 10 samples were found positive for *bcr-abl* fusion, a hallmark of chronic myeloid leukemia. It is characterized by an increase in white blood cell number and unregulated growth of

predominantly myeloid cells in the bone marrow. Bcr-Abl chromosomal abnormality has been reported for CML previously (Breen and Madiano, 2008; Marino *et al.*, 2017). This mutation in a dog is named as the "Raleigh" chromosome (Breen and Madiano, 2008; Marino *et al.*, 2017). In a healthy dog, *bcr* and *abl* gene are located on chromosome 26 and chromosome 9, respectively (Marino *et al.*, 2017). In CML, *abl* gene gets translocated and fuse with *bcr* gene. This hybrid gene increases the activity of tyrosine kinase. Normally, tyrosine kinase helps in cell growth and differentiation, however, in CML, increased tyrosine kinase activity results in abnormal cell growth. As per this method, an amplicon of 305 bp should be generated as has been shown for one CML positive sample, the formation of this amplicon is suggestive of a successful production of functional *bcr-abl* gene fusion. These results were similar to as documented by Radich *et al.* (2001). However, additional bands of lower molecular weight between 50 to 100 bp were also seen in CML-positive samples. This could have been either due to truncated non-functional cross-over translocation or non-specific primer binding which should be investigated by obtaining sequencing data.

In comparison of other animal species, neoplasms of various types are common in dogs. So, development of better cancer diagnostics, particularly nucleic acids based, in dogs is very important for better therapeutic interventions and management of dogs suffering from cancers. In the present study, we are able to successfully diagnose lymphoma, chronic myeloid leukemia and mast cell tumor in dogs using PCR. However, there is still a need to conduct more studies on clinical cases similar to ours to establish the usefulness of nucleic acid-based assays in cancer diagnosis.

Conflict of interest: The authors declare that they have no competing interests.

Author's contribution: SK: Methodology, investigation, analysis, writing of original draft; SV: Methodology, investigation, editing and proof reading of draft; GS: Methodology, proof reading of draft; AK: Methodology, sample collection, investigation; KD: Sample collection, editing and proof reading of draft; MS: Methodology, editing and proof reading of draft.

Data availability statement: The data that support the findings of this study are available in this paper.

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