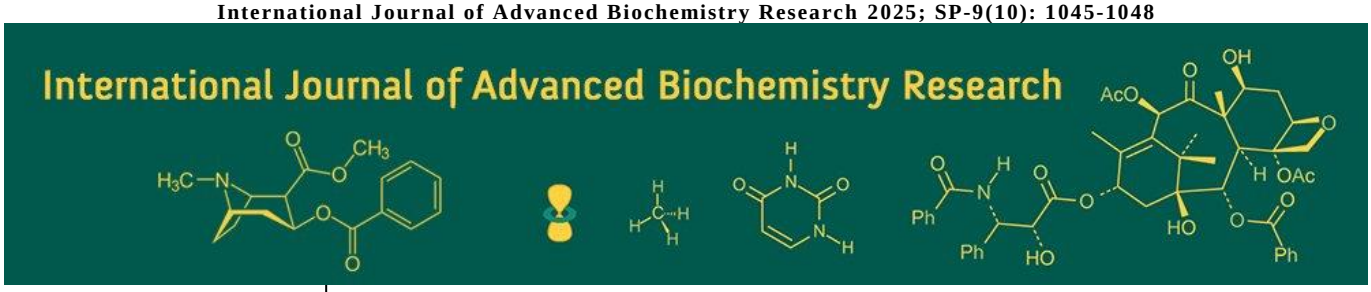

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Comparative evaluation of nested PCR and conventional PCR for detection of sVEGFR-2 gene in canine mammary Tumors

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Abstract
Angiogenic biomarkers play important role in tumor detection. Soluble vascular endothelial growth factor-2 (sVEGFR-2) is one such marker and accurate detection of s-VEGFR-2 is crucial in tumor biology that help in early cancer diagnosis. Conventional PCR often fail to amplify low-copy transcripts which leads to false negatives. This study compares the performance of conventional PCR versus nested PCR to detect sVEGFR-2 in canine mammary tumors and is tallied with cDNA concentration on detection success. From histopatholoically confirmed thirty mammary tumor samples, RNA was extracted, reverse transcribed and conventional PCR as well as nested PCR was performed. Success of the amplification was compared in both the types of PCR and cDNA concentration was analysed relative to PCR positivity. A 546 bp amplicon was produced in 21 out of 30 (70%) tumor samples with conventional PCR, however, nested PCR produced 126 bp amplicon in 28 out of 30 (93.3%) tumor samples with seven additional cases that were negative with conventional PCR. Conventional PCR positives shown higher cDNA concentrations (mean 1024.3 ng/µl); whereas, nested PCR positives expanded detection to samples with lower template concentrations (mean 975.6 ng/µl). Nested PCR displayed superior sensitivity and specificity over conventional PCR. Nested PCR detected sVEGFR-2 transcripts from samples with cDNA concentrations.

Keywords: sVEGR-2, canine mammary tumors, conventional PCR, nested PCR

1. Introduction
Canine mammary tumors (CMTs) are the most common tumors even in the latest observations, in intact (non-spayed) female dogs (Goldschmidt *et al.*, 2017; Raghavendra *et al.*, 2021) [1, 2]. Tumors require more blood supply and enhanced cell proliferation which is supported by angiogenesis (Nistala *et al.*, 2021) [3] and vascular endothelial growth factor (VEGF) pathways play critical role in angiogenesis process. The pro-angiogenic effects of VEGF are mediated majorly through VEGFR-2 (Olsson *et al.*, 2006) [4]. sVEGFR-2 is a decoy receptor that confiscates VEGF and confines angiogenesis (Koch *et al.*, 2011) [5]. Quantifying the expression of sVEGFR-2 can provide insights into tumor angiogenic status and also this can be a potential therapeutic entity. Molecular detection of sVEGFR-2 in early-stage tumors can be technically challenging as the transcript levels of the molecule might be very low. Conventional PCR though widely used, fails to positively amplify such low concentration targets (Hafiz *et al.*, 2005) [6]. Nested PCR is a two-step amplification with outer and inner primer sets which significantly improves sensitivity and specificity. It selectively re-amplifies the target of the first round amplicon (Kwok and Higuchi, 1989) [7]. The present study focussed on comparison of conventional PCR and nested PCR for detecting sVEGFR-2 transcripts in CMTs. Their relationship with cDNA concentration was analysed and documented.

2. Materials and Methods
Canine mammary tumor samples (n = 30) were collected from clinical cases reported at veterinary hospitals in various places like Hyderabad, Bangalore, Visakhapatnam and Gannavaram, India. The same samples were made as sub-sets for both histopathology and molecular analysis.

Tumors were graded using histopathological studies according to WHO guidelines as reported by Goldschmidt *et al.* (2011) [8]. Total RNA from the tumor tissue samples were extracted using TRIzol method, as put forth by Chomczynski and Sacchi, (1987) [9]. The RNA after quality and quantity checks was reverse-transcribed. A 546 bp fragment of sVEGFR-2 was targeted and amplified by using pre-published primers (Table 1) under PCR conditioned mentioned in Table 2. Diluted (1:100) product from conventional PCR was used as template for nested PCR using another set of pre-published internal primers targeting a 126 bp region (Table 3) under PCR conditions shown in Table 4. cDNA concentration data for these tumor samples were sorted and mapped to PCR results.

3. Results

Comparison of both the PCR results were shown in Figure 1. Conventional PCR produced 546 bp product in 21 samples out of 20 (70%) (Figure 2), however, nested PCR amplified a 126 bp product in 28 tumor samples out of 30 (93.3%), including seven that were expressed as negative by conventional PCR (Figure 3). Bands formed by nested PCR were sharper, more distinct and showed negligible non-specific products compared to conventional PCR. Tumor cDNA concentration ranged from 430.1 to 1459 ng/µl. Conventional positives had a mean concentration of 1024.3 ng/µl compared to 551.6 ng/µl in conventional negatives. Nested PCR extended detection to lower-concentration samples (mean 975.6 ng/µl). Distribution of samples and cDNA concentration by histological grade were mentioned in Table 5. Table 6 summarizes cDNA concentration statistics for each group. Figure 4 illustrate the tumor cDNA concentration distribution by conventional and nested PCR attributes.

4. Discussion

The present study validates that nested PCR significantly enhances the detection of sVEGFR-2 amplicons in comparison to conventional PCR, more particularly in tumor samples with lower cDNA concentrations. Our result conveyed that where conventional PCR failed to detect sVEGFR-2 in samples with mean cDNA concentrations as lower as 550 ng/µl, nested PCR successfully amplified such intermediate-concentration samples and thereby increasing overall detection rate to 93.3%. These findings are backed by the reports of previous workers (Hafiz *et al.*, 2005; Kwok and Higuchi, 1989) [6, 7] who noted that nested PCR can lower the detection limit in comparison to conventional PCR.

The superior performance of nested PCR over conventional PCR can be attributed to its two-round amplification, which selectively augments the target amplicon thereby reducing the non-specific background noise (Kwok and Higuchi, 1989) [7]. In our work this was evident by sharper and more distinct bands in our nested PCR gels. More importantly, Grade I tumors that were regarded negative for the specific cDNA sequence by conventional PCR were detected successfully as positive by nested PCR technique. This underscore the value of nested PCR in early-stage disease diagnosis where the transcript levels may be low.

Our finding put forth that nested PCR is a superior molecular detection tool of sVEGFR-2 compared to conventional PCR, especially in early-stage tumors or whrn RNA quality is not up to the mark. However, stricter lab practices are mandatory for its higher sensitivity to avoid false positives.

Table 1: Conventional PCR primers

Gene	Accession no. (NCBI)	Product size	Oligo size	Primers
sVEGFR-2-FP	NM_001048024.1	546	20	5’ CACGGAGTTGAGCTGTCTGT3’
sVEGFR-2-RP			20	5’ TCACGTGGCTCTGTTTCTCC3’

Table 2: Cycling conditions for conventional PCR

Step No.	Step	Temperature/Time
1	Initial denaturation	95 °C/10 min
2	Denaturation	95 °C/15 sec
3	Annealing	60 °C/30 sec
4	Extension	72 °C/40 sec
5	No. of cycles from step 2 to 4	35
6	Final Extension	72 °C/10 min

Table 3: Nested PCR primers

Gene	Accession no. (NCBI)	Product size	Oligo size	Primers
sVEGFR-2-FP	NM_001048024.1	126	20	5’ GACCAAGGGTGGTACACCTG 3’
sVEGFR-2-RP			20	5’ GGCTTCCACCGAAGATTCCA 3’

Table 4: Cycling conditions for nested PCR

Step No.	Step	Temperature/Time
1	Initial denaturation	95 °C/10 min
2	Denaturation	94 °C/1 min
3	Annealing	53 °C/1 min
4	Extension	72 °C/1 min
5	No. of cycles from step 2 to 4	35
6	Final Extension	72 °C/10 min

Table 5: Distribution of samples and cDNA concentration by histological grade

Group	n	Mean±SD (ng/µl)	Range (ng/µl)
Grade I (GI)	10	796.4±301.9	430.1-1459.0
Grade II (GII)	10	927.1±332.9	452.4-1410.0
Grade III (GIII)	10	883.1±275.2	456.1-1342.9
Total (Tumor)	30	868.9±293.1	430.1-1459.0

Table 6: Summary statistics for tumor cDNA concentration by PCR detection status.

Group	n	Mean (ng/µl)	Median (ng/µl)	Std Dev (ng/µl)
Conventional positive	21	1024.3	978.9	256.8
Conventional negative	9	551.6	556.5	47.5
Nested positive	28	975.6	978.3	263.0
Nested negative	2	484.3	484.3	41.7

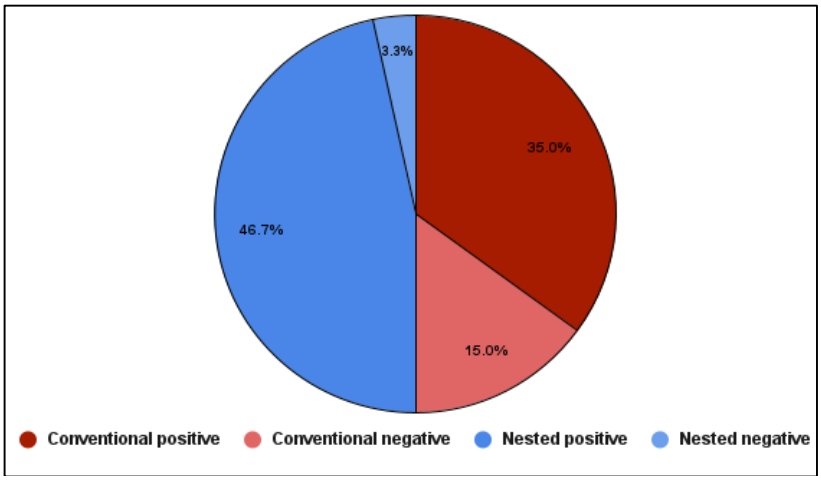

Fig 1: Distribution of results: Conventional Vs Nested PCR

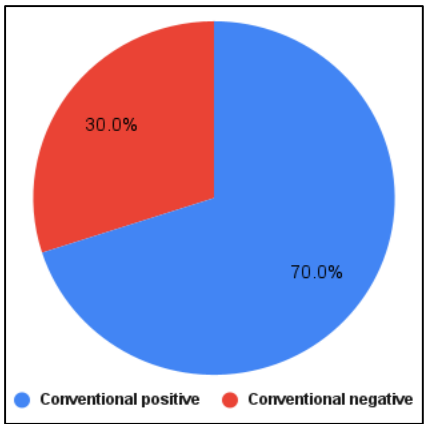

Fig 2: Distribution of Conventional PCR results

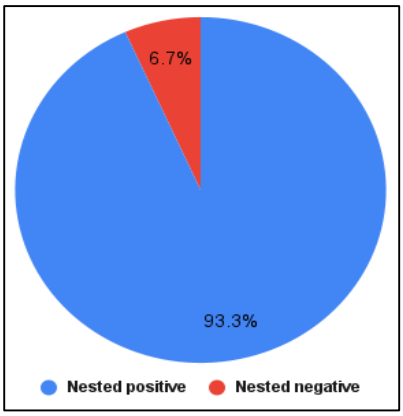

Fig 3: Distribution of Nested PCR results

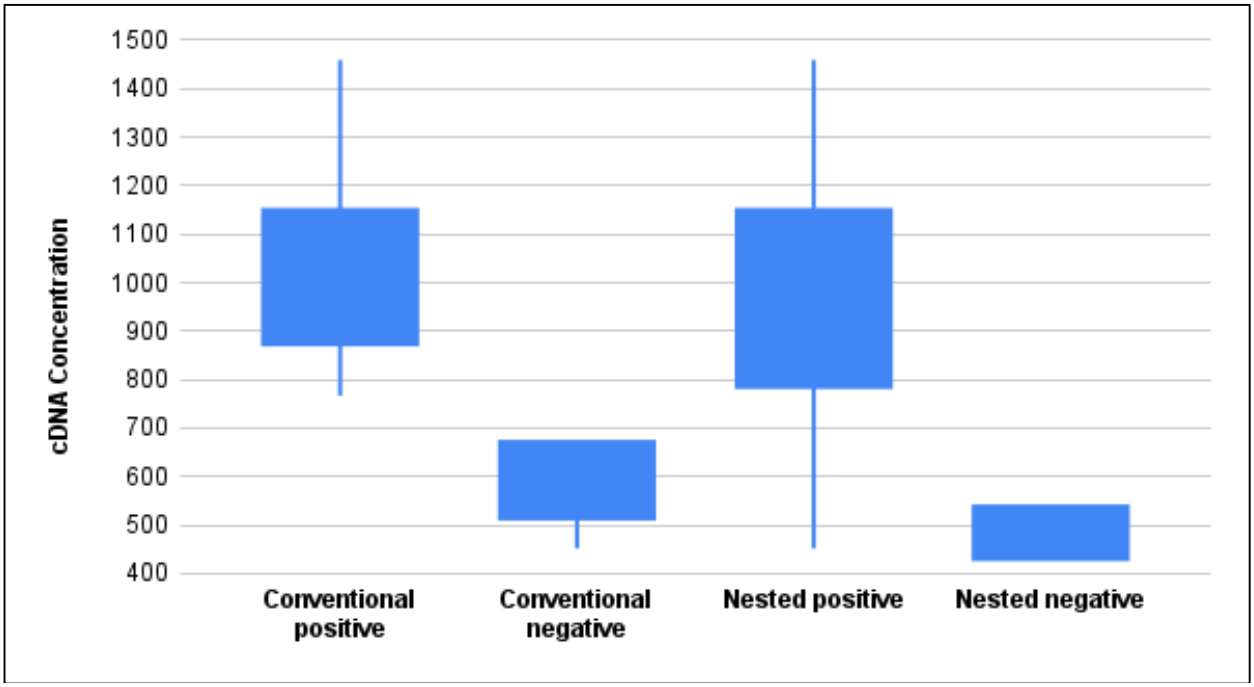

Fig 4: Tumor cDNA concentration distribution by conventional and nested PCR attributes

5. Conclusion

In this present study of ours, we noticed that nested PCR outperformed conventional PCR for the detection of sVEGFR-2 in canine mammary tumors, covering detection into lower cDNA concentration ranges and detecting samples missed by conventional PCR.

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