RESEARCH ARTICLE

Molecular Characterisation of Trefoil Factor 1 from Cases of Canine Mammary Tumour

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Abstract

Canine mammary tumours (CMT) are among the most common neoplasms in female dogs and exhibit variable biological behaviour, necessitating the identification of reliable molecular biomarkers. Trefoil Factor 1 (*TFF1*) has been studied extensively in human cancers and has been found to be an important prognostic marker associated with tumour growth and progression. The present study was aimed to characterise *TFF1* in canine mammary tumours tissues collected from clinical cases (n=10). Histopathological examination revealed diverse tumour patterns, including tubulopapillary carcinoma, carcinoma in situ, and stromal desmoplasia, indicating varying degrees of malignancy. Immunohistochemical analysis demonstrated variable cytoplasmic *TFF1* expression in neoplastic epithelial cells, ranging from strong to weak immunoreactivity among different tumour subtypes, indicating heterogeneous protein expression within canine mammary tumours. At the molecular level, the canine *TFF1* gene was successfully amplified, cloned and sequenced. Sequence analysis showed 100% nucleotide identity with the published canine *TFF1* sequence, while phylogenetic analysis demonstrated a high degree of conservation among mammalian species, particularly within the Carnivora lineage, highlighting its evolutionary significance.

Key words: Canine mammary tumour, Gene cloning, Phylogenetic analysis, Trefoil Factor 1 (*TFF1*).

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Introduction

Canine mammary tumours (CMTs) are among the most common neoplasms affecting intact female dogs and constitute a major concern in veterinary oncology. Approximately 50-70% of mammary tumours in dogs are malignant, with incidence increasing significantly in older, non-spayed females (Sorenmo *et al.,* 2011). Similar to human breast cancer, canine mammary tumours are characterised by marked histopathological heterogeneity, invasive potential, hormonal influence, and metastatic behaviour. Due to these similarities, spontaneous canine mammary tumours have emerged as valuable comparative models for studying the molecular mechanisms, progression, and therapeutic targets of human breast cancer (Liu *et al.,* 2014). A recent study showed significant parallels in the expression patterns of *ER*, *PR*, *HER-2*, and *Ki-67* between CMT and human breast cancer (Narad *et al.,* 2026). The two-year survival rate for female dogs with malignant mammary tumours ranges from 25% to 40% and varies with tumour type, grade, metastasis, stage, and treatment (Cavalcanti and Cassali, 2006; Cassali *et al.*, 2020). Prognostic factors based on clinical, pathological, and biological characteristics are essential for predicting disease outcome, although validated biomarkers in veterinary oncology remain limited and are often assessed by immunohistochemistry at a single time point (de Nardi *et al.*, 2007; Cassali *et al.*, 2020; Narad *et al.,* 2026).

The trefoil factor (*TFF*) family, comprising *TFF1, TFF2*, and *TFF3*, consists of small cysteine-rich peptides with conserved trefoil domains that confer stability and resistance to harsh conditions (Chinery *et al.*, 1996; Wong *et al.*, 1999). *TFFs* are involved in mucosal protection, epithelial repair, and regulation of proliferation, apoptosis, and migration (Wright *et al.,* 1993). Aberrant expression of *TFFs* has been associated with cancer progression; for instance, *TFF3* overexpression correlates with tumour size, metastasis, and poor prognosis in breast and liver cancers (Pandey *et al.*, 2014; Shang and Qian, 2014). Conversely, loss of *TFF1* expression in gastric cancer is linked to gene mutation or promoter hypermethylation (Park *et al.*, 2000; Fujimoto *et*

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al., 2000). Experimental and clinical studies suggest that *TFF1* functions as a tumour suppressor, whereas *TFF3* may act as a prognostic marker, highlighting the diverse and context-dependent roles of *TFFs* in cancer biology (Buache *et al.*, 2011; Mhawech-Fauceglia *et al.*, 2013). This paper reports the molecular characterisation of trefoil factor 1 from cases of canine mammary tumour.

The objective of the present study was not to identify a novel *TFF1* sequence, mutation, or transcript variant, but to comprehensively characterize tumour-derived canine *TFF1* at both the molecular and protein levels. Specifically, we evaluated the immunohistochemical expression of *TFF1* in canine mammary tumour tissues, amplified, cloned, and sequence-confirmed the canine *TFF1* gene and performed phylogenetic analysis to assess its evolutionary conservation. This integrated approach provides molecular and protein-level validation of *TFF1* and establishes a foundation for future functional studies, recombinant antigen production, antibody generation, and biomarker assay development in canine mammary tumours.

Materials and Methods

Clinical Sample Collection

All experimental procedures were approved by the Institute Animal Ethics Committee (Nos. F.26-1/2015-16/JD.R and 26-1/2023-24/JD(R)/IAEC) and conducted in compliance with the CPCSEA guidelines. Clinical samples (n=10) were obtained from animals presented with suspected mammary gland tumours at the Referral Veterinary Polyclinic of the ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, India. These cases included 4 Labrador retriever, 2 German shepherd, 1 Rottweiler, and rest 3 Desi or non-descript female dogs, aged 3 to 8 years, weighing 26 to 46 kg, majority with thoracic gland involvement of 3 to 8 months duration size 2.5 x 3.0 to 5.5 x 6.0 cm.

Histopathological Evaluation of Canine Tumour

Excised mammary tumour tissues were fixed in 10% neutral buffered formalin for approximately 24 h at room temperature. After fixation, samples were dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax according to routine histological procedures. Paraffin-embedded blocks were sectioned at 4 µm thickness and placed on glass slides for microscopic examination. For histopathological evaluation, sections were stained with haematoxylin and eosin (H&E). Histopathological diagnoses and tumour classification were performed according to the criteria of Goldschmidt *et al.* (2011). The mitotic index was determined by counting mitotic figures in ten randomly selected high-power (40x) fields.

Immunohistochemical Detection of *TFF1*

Immunohistochemical analysis of Trefoil Factor 1 (*TFF1*) was performed on formalin-fixed, paraffin-embedded canine mammary tumour tissues. Tissue sections (4 µm) were mounted on silane-coated glass slides and incubated overnight at 37°C to ensure proper adhesion. Sections were deparaffinized in xylene, rehydrated through graded ethanol, and treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed using Abcam antigen retrieval buffer according to the manufacturer's instructions. After blocking non-specific binding sites, the sections were incubated overnight at 4°C with a rabbit polyclonal anti-*TFF1* antibody (Invitrogen, Cat. No. AC4652898A; 1:200 dilution). Immunoreactivity was detected using a horseradish peroxidase (HRP)-conjugated detection system with 3,3′-diaminobenzidine (DAB) as the chromogen. Sections were counterstained with Mayer's hematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted with DPX mounting medium. The stained sections were examined under an Olympus BX41 light microscope, and representative images were captured and analysed using Olympus digital imaging software.

Isolation of Total RNA and Synthesis of Complementary DNA

RNA was extracted from mammary tumour samples using TRIzol reagent (Invitrogen) according to standard phenol-chloroform extraction procedures. RNA concentration and purity were assessed spectrophotometrically using a NanoDrop system (Thermo Fisher Scientific). Samples with A260/A280 ratios between 1.8 and 2.0 were considered suitable for downstream applications. RNA integrity was verified by electrophoresis on 1.5% agarose gel to confirm intact ribosomal RNA bands. Complementary DNA (cDNA) was synthesised from 1 µg of total RNA using a reverse transcription kit (High-Capacity cDNA Reverse Transcription Kit; Thermo Fisher Scientific) in a 20 µL reaction volume, following the manufacturer's recommendations. Synthesised cDNA was stored at −20 °C until further use.

PCR Amplification of *TFF1* Gene

TFF1-specific primers were designed using NCBI Primer-BLAST, PrimerQuest Tool (Integrated DNA Technologies), and OligoAnalyzer (IDT) to ensure optimal GC content (40–60%), melting temperature (58–62°C), and minimal secondary structure formation. The chemically synthesized primers were procured from Life Science (P) Ltd., and PCR conditions were optimized using gradient PCR on a Bio-Rad Thermal Cycler. The forward primer (25 bp; 5′-AAGGTACCAGGAGCAGAGAGGACG-3′) and reverse primer

(27 bp; 5′-AAGGATCCAAGGGACACTCCTCTTCGG-3′) were designed to provide optimal melting temperature, GC content, target specificity, and minimal secondary structure formation. The slight difference in primer length was required to achieve comparable melting temperatures and efficient amplification while incorporating *KpnI* and *BamHI* restriction sites at the 5′ ends for directional cloning. PCR amplification was performed in a thermal cycler under optimised melting temperature (60-65°C), and facilitate the incorporation of restriction enzyme recognition sites for molecular cloning.

PCR amplification was performed in a thermal cycler using Phusion™ Plus High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA). Each 100 µL reaction contained 20 µL of 5× Phusion™ Plus Buffer, 1.0 µL Phusion™ Plus DNA Polymerase, 2.5 µL each of forward and reverse primers (20 pmol), 2.0 µL of 10 mM dNTP mix, 5.0 µL of template cDNA, and 67.0 µL of nuclease-free water (Qiagen, Germany). The PCR conditions consisted of an initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. The proofreading activity of Phusion™ Plus DNA polymerase ensured high-fidelity amplification suitable for downstream cloning and sequencing. The amplified products were analysed on 1.5% agarose gel in 1× TAE buffer using a 50-bp DNA ladder to confirm the expected amplicon size.

Molecular Cloning of the Canine *TFF1* Gene

The amplified products were purified with the MinElute PCR Purification Kit (Qiagen) following standard protocols. The purified fragments were then digested with *KpnI* and *BamHI* restriction endonucleases (New England Biolabs) and ligated into the pH6HTN His6 HaloTag T7 expression vector (Promega) using T4 DNA ligase. The resulting recombinant constructs were introduced into *Escherichia coli* DH5α competent cells via transformation and cultured on LB agar plates containing 100 µg/mL ampicillin.

Phylogenetic Analysis

The nucleotide sequence of the cloned canine *TFF1* gene was subjected to similarity analysis using the BLASTn program available at the National Center for Biotechnology Information (NCBI). Homologous *TFF1* nucleotide sequences representing diverse mammalian species were retrieved from the GenBank database. Multiple sequence alignment was performed using the MUSCLE algorithm implemented in MEGA version 7.0 (Edgar, 2004; Kumar *et al.,* 2016). Poorly aligned terminal regions and ambiguous positions were manually trimmed before phylogenetic analysis to obtain a high-quality alignment.

The best-fit nucleotide substitution model was selected using the model selection function in MEGA 7 based on the lowest Bayesian Information Criterion (BIC) value. A phylogenetic tree was reconstructed using the Maximum Likelihood (ML) method with 500 bootstrap replicates to assess the robustness of the inferred phylogenetic relationships. The tree was rooted using [Outgroup species] and bootstrap values (%) were displayed at the corresponding nodes. The resulting phylogeny was used to evaluate the evolutionary relationship and sequence conservation of canine *TFF1* with homologous mammalian sequences.

Results and Discussion

Gross Morphological Features of CMT

Ten female dogs with clinically diagnosed mammary tumours were included in the study. The affected animals ranged from 3 to 8 years of age and had body weights ranging from 26.0 to 46.0 kg. Tumours were observed in different mammary gland regions, with the thoracic mammary glands (M1–M2) being the most frequently affected site (7/10; 70%), followed by the abdominal mammary glands (M3–M4) (2/10; 20%) and the inguinal mammary gland (M5) (1/10; 10%). One abdominal tumour represented a recurrent mammary carcinoma.

The tumour masses varied considerably in size, ranging from 1.5 × 1.4 cm to 5.5 × 6.5 cm. Benign tumours generally presented as small, well-circumscribed nodules measuring approximately 1.5–2.8 cm, whereas malignant tumours tended to be larger, with dimensions ranging from 3.5 × 4.0 cm to 5.5 × 6.5 cm. The recurrent mammary carcinoma was the largest lesion (5.5 × 6.5 cm).

Gross examination of canine mammary tumours (CMT) demonstrated characteristic clinical and morphological alterations (Fig. 1). The affected animal presented with a prominent, firm mammary mass exhibiting irregular margins and visible surface ulceration, accompanied by focal haemorrhagic areas, indicating advanced tumour progression (Fig. 1A). The tumour appeared adherent to the surrounding tissue, suggesting possible local invasion. Post-surgical evaluation of the excised tumour revealed an irregular, multilobulated mass with a fleshy and heterogeneous appearance (Fig. 1B).

Histopathological Characterisation of CMT

Histopathological examination of the ten canine mammary tumour (CMT) cases revealed an equal distribution of benign (5/10; 50%) and malignant (5/10; 50%) neoplasms. Among the benign tumours, mammary adenoma was the most frequently diagnosed lesion (4/5; 80%), comprising simple mammary adenoma (n=1), tubular adenoma (n=1), papillary

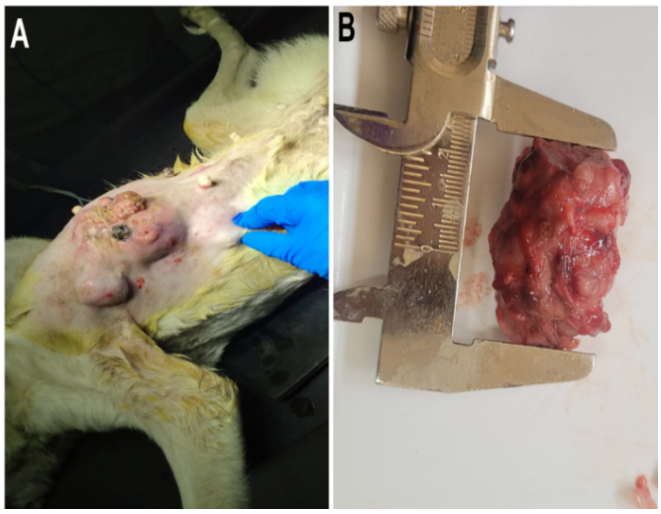


Fig. 1: Gross morphology of canine mammary tumours (CMT). (A) Clinical presentation of a dog showing a firm, enlarged mammary mass. (B) Measurement of an excised tumour mass using a digital Vernier calliper to determine tumour dimension.

adenoma (n=1), and simple adenoma (n=1). The remaining benign case was diagnosed as fibroadenoma (1/5; 20%). The malignant tumours exhibited greater histopathological heterogeneity. One case (20%) was diagnosed as carcinoma in situ (Grade III), while four cases (80%) were invasive mammary carcinomas. These included tubulopapillary carcinoma (Grade I) (n=), solid carcinoma (Grade III) (n=2), including one recurrent tumour, and comedocarcinoma (Grade III) (n=1). Overall, four of the five malignant tumours (80%) were classified as Grade III, indicating a predominance of poorly differentiated, high-grade lesions, whereas only one tumour (20%) was classified as Grade I. These findings demonstrate the presence of diverse benign and malignant mammary tumour subtypes, with high-grade carcinomas representing the predominant malignant phenotype in the studied cohort.

Histopathological examination of canine mammary tissues revealed distinct morphological features corresponding to different tumour patterns. Tubulopapillary carcinoma, with prominent papillary and arborising structures supported by fibrovascular cores. The neoplastic epithelial cells exhibited marked pleomorphism, hyperchromatic nuclei, increased nuclear-to-cytoplasmic ratio, and frequent mitotic Figures, indicating high proliferative activity (Fig. 2A-B). Carcinoma in situ, where well-circumscribed neoplastic cell clusters remained confined within the basement membrane, displayed moderate anisocytosis and anisokaryosis, suggesting early neoplastic transformation without stromal invasion (Fig. 2C). Tumour microenvironment was characterised by notable inflammatory cell infiltration along with vascular congestion, reflecting an active stromal response and possible tumour-associated inflammation (Fig. 2D).

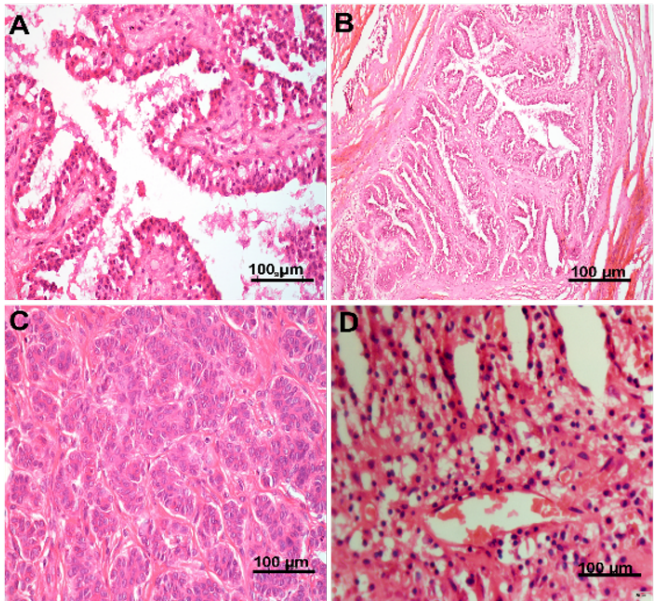


Fig. 2: Histopathological features of canine mammary tumour. (A-B) Tubulopapillary carcinoma showing papillary arborescent structures with fibrovascular cores and pleomorphic epithelial cells exhibiting hyperchromatic nuclei and high nuclear-to-cytoplasmic ratio with frequent mitoses. (C) Carcinoma in situ is characterised by well-demarcated neoplastic nodules confined within the basement membrane with moderate anisocytosis and anisokaryosis. (D) Tumour stroma showing inflammatory cell infiltration and vascular congestion (H&E, ×100).

Immunohistochemical Expression of *TFF1*

Immunohistochemical analysis revealed variable expression of *TFF1* in canine mammary tissue samples. Figure 3A shows strong positive cytoplasmic immunoreactivity for *TFF1* in neoplastic epithelial cells, particularly within tumour glands and surrounding invasive areas. Intense brown staining (indicated by arrows) confirms high *TFF1* expression in tumour cells, suggesting active involvement in tumour progression. Figure 3B demonstrates moderate *TFF1* expression, characterized by diffuse cytoplasmic staining in glandular epithelial cells. The staining intensity was lower compared to Figure A but remained clearly distinguishable, indicating intermediate expression levels of *TFF1* within the tumour tissue. In contrast, Figure 3C exhibits negative to weak *TFF1* immunostaining, with minimal or no detectable brown coloration in the stromal and epithelial components. This pattern represents low or absent *TFF1* expression, comparable to non-neoplastic or low-expressing tissues.

PCR Amplification of the Canine *TFF1* Gene

Amplification of the canine *TFF1* gene generated a distinct 271 bp fragment, as confirmed by agarose gel

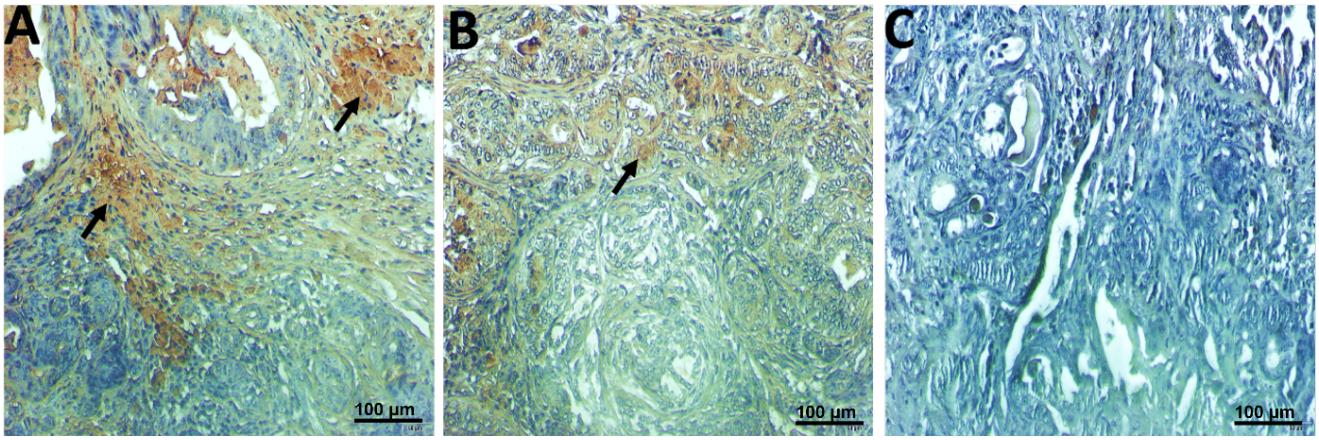


Fig. 3: Immunohistochemical expression of *TFF1* in canine mammary tissue. Representative photomicrographs showing cytoplasmic immunoreactivity of *TFF1* in canine mammary tissues. (A) Strong positive *TFF1* expression in neoplastic epithelial cells with intense brown cytoplasmic staining (arrows). (B) Moderate *TFF1* immunoreactivity showing diffuse cytoplasmic staining in tumour glandular epithelium. (C) Weak to negative *TFF1* expression with minimal or absent staining in epithelial and stromal components. (Immunohistochemistry, DAB chromogen, hematoxylin counterstain; original magnification ×100).

electrophoresis analysis. Specific amplification bands corresponding to the expected amplicon size were observed in lanes L1 and L5. In contrast, no amplification was observed in the negative control, confirming the specificity of the reaction (Fig. 4) indicating successful amplification of the target *TFF1* gene from canine mammary tumour-derived cDNA. The amplified *TFF1* PCR products were subsequently utilised for downstream molecular characterisation.

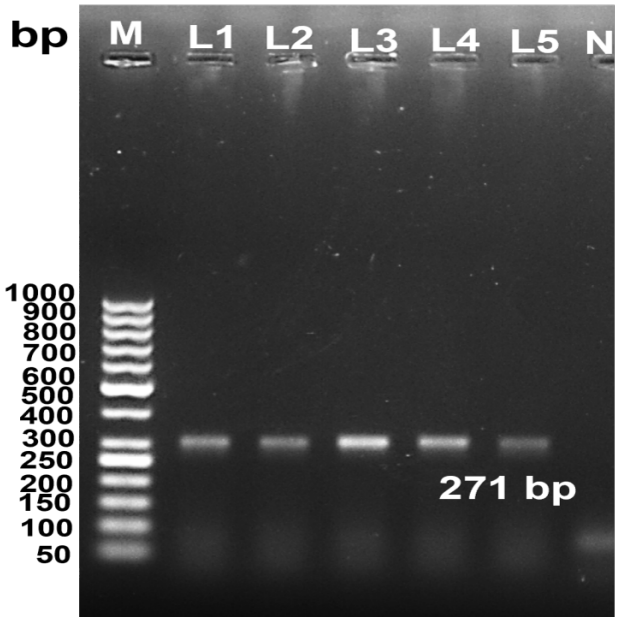


Fig. 4: Agarose gel electrophoresis showing PCR amplification of the canine *TFF1* gene from canine mammary tumour-derived cDNA samples. Lane M: 50 bp DNA ladder (puregene); Lanes L1-L5: amplified *TFF1* gene products showing the expected amplicon size of 271 bp; Lane N: negative control showing no amplification

Molecular Cloning of the Recombinant Canine *TFF1* Gene

Successful transformation of *E. coli* DH5α cells with the recombinant *TFF1* plasmid was confirmed by the appearance of numerous well-isolated colonies on selective agar plates (Fig. 5A), indicating efficient uptake of the plasmid construct. Restriction enzyme digestion analysis further validated the cloning, with the recombinant plasmid yielding two distinct fragments corresponding to the ~5.9 kb plasmid backbone and the ~271 bp *TFF1* insert (Fig. 5B). The presence of these expected bands confirmed successful insertion of the target gene into the vector.

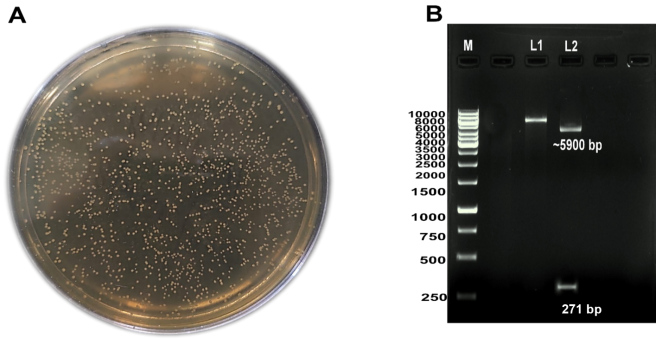


Fig. 5: Transformation and confirmation of recombinant *TFF1* plasmid in *E. coli* DH5α. (5A) Shows successful transformation of *E. coli* DH5α cells with the recombinant *TFF1* plasmid. (5B) Depicts restriction enzyme (RE) digestion analysis of the isolated recombinant plasmid, confirming the presence of the *TFF1* insert. Lane M represents 1 kb DNA ladder (puregene), while lanes L1 uncut plasmid and L2 show the release of the ~271 bp *TFF1* insert along with the ~5.9 kb plasmid backbone, validating successful cloning.

Phylogenetic Analysis of *TFF1* Genes across Mammalian Species

Phylogenetic analysis of the canine *TFF1* nucleotide sequence demonstrated a high degree of evolutionary conservation among mammalian species. The phylogenetic tree resolved the sequences into distinct taxonomic clades. The canine *TFF1* sequence (*Canis lupus familiaris*) clustered closely with other members of the family Canidae, including *Canis lupus baileyi* and *Canis aureus*, indicating close evolutionary relatedness within the genus *Canis*. This cluster was further associated with other canids, namely *Vulpes vulpes*, *Vulpes lagopus*, and *Nyctereutes procyonoides*, with moderate to high bootstrap support.

The canid clade formed part of a broader carnivore lineage comprising members of the Felidae, including *Felis catus, Panthera pardus, Neofelis nebulosa, Lynx canadensis, Prionailurus bengalensis, Prionailurus viverrinus, Leopardus geoffroyi*, and *Herpailurus yagouaroundi*, as well as the Hyaenidae species *Hyaena hyaena* and *Crocuta crocuta*. In contrast, the primate sequences (*Homo sapiens*, *Pan paniscus*, and *Gorilla gorilla*) formed a separate clade, reflecting greater evolutionary divergence from the canine *TFF1* gene (Fig. 6).

The present study underscores the relevance of Trefoil Factor 1 (*TFF1*) in CMT based on integrated histopathological, immunohistochemical, and molecular analyses. Histological findings confirmed malignant features such as tubulopapillary carcinoma, carcinoma in situ, and stromal desmoplasia, consistent with established indicators of tumour aggressiveness (Dutra *et al.,* 2008; Goldschmidt *et al.,* 2011; Cassali *et al.,* 2020). The presence of inflammatory infiltration further highlights the role of the tumour microenvironment in disease progression (Clemente *et al.,* 2010; Sánchez-Céspedes *et al.,* 2021). Immunohistochemical analysis revealed heterogeneous cytoplasmic *TFF1* expression in neoplastic epithelial cells, with strong immunoreactivity in some tumour regions and weaker expression in others, indicating variable *TFF1* expression within canine mammary tumours. Similar dysregulation has been reported in human cancers, where *TFF1* is linked to epithelial proliferation and progression, and may interact with estrogen receptor pathways (Wright *et al.,* 1993; Wong *et al.,* 1999; Mhawech-Fauceglia *et al.,* 2013).

The role of *TFF1* appears context-dependent, functioning as a tumour suppressor in some cancers (Park *et al.,* 2000; Fujimoto *et al.,* 2000) while contributing to tumour progression in others (Pandey *et al.,* 2014; Shang and Qian, 2014). Experimental evidence further supports its protective role in epithelial homeostasis (Buache *et al.,* 2011). Molecular analyses confirmed successful cloning

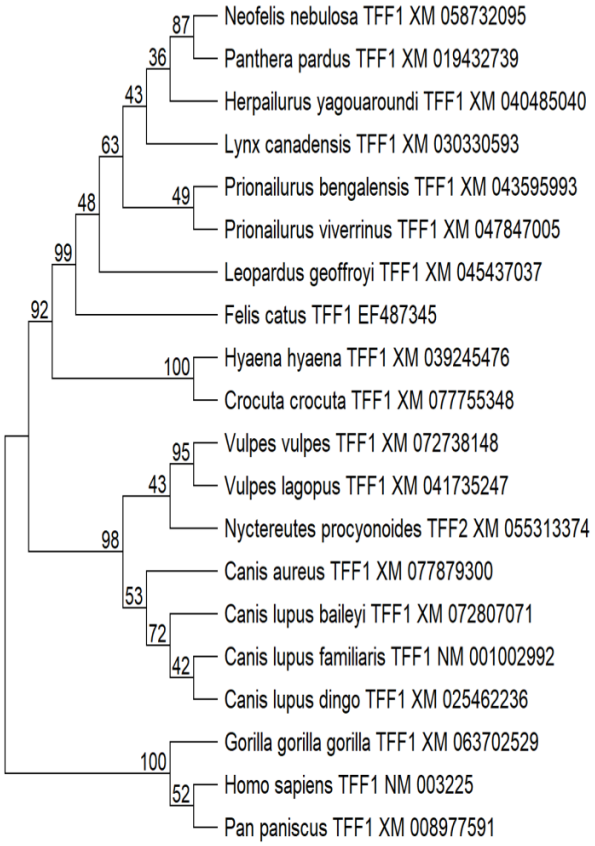


Fig. 6: Phylogenetic tree of the canine *TFF1* gene showing its evolutionary relationship with homologous mammalian *TFF1* sequences. Sequences were aligned using MUSCLE, and the tree was constructed in MEGA 7 using the Neighbour-Joining method with 500 bootstrap replicates. Bootstrap values (%) are indicated at the branch nodes.

supporting its potential utility in diagnostic applications. Phylogenetic analysis demonstrated high conservation of *TFF1* across species, indicating its fundamental biological role (Chinery *et al.,* 1996; Wong *et al.,* 1999).

Conclusion

The present study provides an integrated characterization of Trefoil Factor 1 (*TFF1*) in canine mammary tumours through histopathological, immunohistochemical, molecular, and phylogenetic analyses. Histopathology revealed diverse benign and malignant tumour subtypes, while immunohistochemistry demonstrated heterogeneous cytoplasmic *TFF1* expression in neoplastic epithelial cells. Molecular analyses confirmed successful amplification, cloning, and sequence characterization of the canine *TFF1* gene, and phylogenetic analysis demonstrated its high conservation among mammalian species. These findings support the potential of *TFF1* as a candidate biomarker in canine mammary tumours and provide a foundation

for future studies. Further validation in larger, clinically characterized cohorts is required to establish its diagnostic and prognostic significance.

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