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Molecular detection and characterization of canine papilloma virus 1 (CPV 1) using early and late gene fragments in dog, Tamil Nadu

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Abstract
Canine papillomavirus causes papillomas and plaques on the skin and mucous membranes of different sites. The present study aimed to identify the genotype of the canine papillomavirus associated with oral papillomatous growth. A six month old male Rottweiler was carried to Veterinary College and Research Institute, Orathanadu with a history of wart like growths protruding from the oral cavity. The growths suspected of papilloma from the oral mucosa were removed surgically for detection of canine oral papillomavirus (COPV) by PCR. The PCR was carried out by amplifying the portion of early (E6, E7) and late genes (L1) of COPV by using respective gene specific primers. Further, nucleotide sequencing and phylogenetic analysis of early (E6, E7) and late genes (L1) revealed that the sequences were closely related with previously reported CPV 1 belonging to the *Lambdapapillomavirus* genus. The present study concluded that the CPV 1 was the cause of papillomatous growths which is mostly associated with non-neoplastic papillomas in the oral cavity.

Keywords: Canine papillomavirus 1, PCR, genotyping, phylogenetic analysis and *Lambdapapillomavirus*

1. Introduction
Papillomavirus belongs to a double-stranded DNA virus that primarily infects keratinocytes which causes benign and malignant lesions in the skin and epithelial lining of oral cavity of various animals as well as humans (Howley and Lowy, 2007) [1]. Canine papillomaviruses were reported to have three genera *viz*. *Lambdapapillomavirus* which consists of CPV 1 and CPV6, *Taupapillomavirus* contains CPV 2, CPV7 and CPV13 and *Chipapillomavirus* included CPV 3, CPV4, CPV5, CPV8, CPV9, CPV10, CPV11, and CPV14 (Bernard *et al*., 2010; Lange and Favrot, 2011; Lange *et al*., 2012; Yuan *et al*., 2012) [2-5]. Canine Papillomaviruses are associated with exophytic and endophyticpapillomas, pigmented plaques and also *in situ* and invasive squamous cell carcinomas (Bernard *et al*., 2010) [2].
Papillomaviruses were reported to have a circular genome with non-enveloped icosahedral capsid which consists of early viral proteins E1 to E7 and late proteins L1 and L2 (Howley and Lowy, 2007) [1]. The early and late proteins were reported to be responsible for viral DNA replication and viral capsid formation as well as assembly of viral DNA respectively. The L1 gene is highly conserved which plays a major role in the classification of papillomaviruses (De Villers *et al*., 2004) [6]. It has been reported that more than 200 new putative Papillomavirus types were characterized based on the variation of L1 gene nucleotide sequences mostly in humans (Bernard *et al*., 2010) [2]. De Villers *et al*. (2004) [6] reported the categories for description of new putative papillomavirus types as genus (less than 60% identities), species (more than 60% identities), type (more than 70% identities), subtype (more than 90% identities) and variant (more than 98% identities). Presently the genomes of 14 canine papillomaviruses (CPVs) types have been reported (Yuan *et al*., 2012; Lange *et al*., 2013) [5, 7].
Canine papillomavirus infection has been diagnosed by gross morphological, histopathological or immunohistochemical methods and PCR. Molecular methods such as PCR are considered as the most specific and sensitive method even short stretches of viral DNA are detected by

sequencing (Lange and Favrot, 2011) [3]. In the present study, CPV1 belonging to the *Lambdapapillomavirus* genus was detected in the oral papillomatous growths by PCR as well as sequencing.

2. Materials and Methods

2.1 Collection of samples

A six month old Rottweiler dog was presented to the Teaching Veterinary Clinical Complex, Veterinary College and Research Institute, Orathanadu with a history of proliferative oral lesions. The oral papillomatous growth from a clinically diagnosed case was surgically removed and collected for molecular diagnosis.

2.2 DNA extraction and PCR

DNA was extracted from the oral wart tissue using Tissue DNA Purification Kit (Qiagen) following the manufacturer's instruction. The obtained DNA was stored at -20 °C until further analysis. Then, Polymerase chain reaction was carried out by amplifying the E6, E7 and L1 gene fragments by using respective gene specific primers as shown in Table 1. The PCR reaction mixture was composed of 12.5 µl of master mix (2x), 1 µl of each forward and reverse primers (10 pmol/ µl), 7.5 µL of deionized water and 3 µl of extracted DNA in a total volume of 25 µl. The PCR program was carried out as previously described by Brandes *et al.* (2009) [8] for amplifying E6, E7 and L1 gene fragments of COPV. The PCR amplicons were electrophoresed in 1.5 percent agarose gel stained with ethidium bromide (0.5µg/ml) and documented under the Gel documentation system. The PCR products were purified by Hiyield plus PCR purification kit (cat#QPP100) following the manufacturer's instruction.

Table 1: Primers used in this study

Target gene	Primer sequence	Size	References
E6	GGCACTGTTATCAATGGAGC CACATAGTTCTTTGTCCGCC	350 bp	Brandes *et al.* (2009) [8]
E7	GATATTGTGCTGACAGAGC AAAGTGACCTGCAGCACACG	204 bp	
L1	CTTGTTTGGGGCTTAAGAGG TGCAGTGTGTACCTGTCCTG	261 bp	

2.3 Sequence analysis

The positive PCR amplicons of E6, E7 and L1 genes of COPV were subjected to sequencing by Sanger dideoxy sequencing method in an automated sequencer. The obtained forward and reverse sequences were aligned using Bio Edit software to obtain consenses sequences using reference sequence retrieved from NCBI data base. Then, multiple sequence alignment was carried out using published sequences of COPV available in the NCBI database by MEGA 11.0 software (Clustal W). The phylogenetic tree was constructed to estimate the relationship between sequences by using the Maximum likelihood algorithm using bootstrap values and distance in MEGA 11.0 software.

3. Results

The DNA extracted from oral growth was positive for the canine oral papillomavirus by PCR. The E6, E7 and L1 gene fragments of COPV were amplified by PCR and yielded approximately 350 bp, 204 bp and 261 bp in length respectively. The PCR amplicons of E6, E7 and L1 genes of COPV are shown in Fig. 1. The amplicons were purified and sequenced for characterization of COPV.

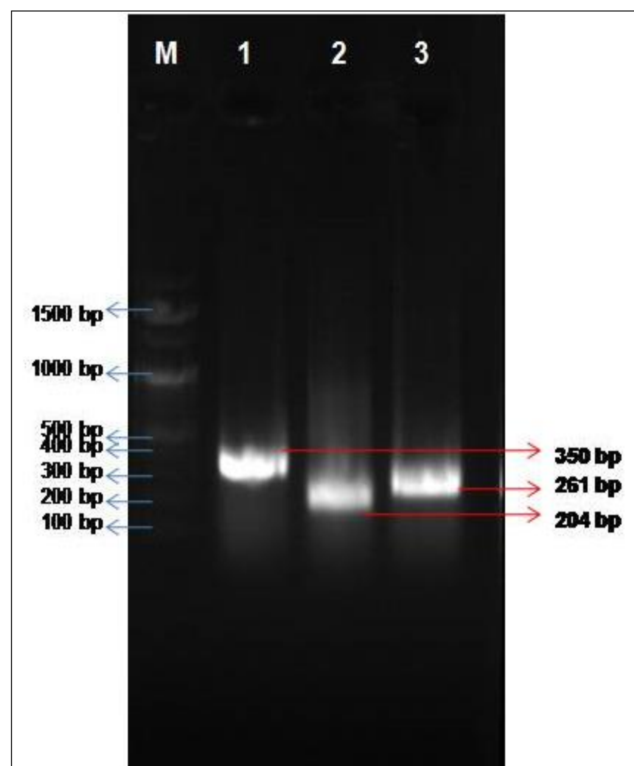

Fig 1: PCR for E6, E7 and L1 gene fragments of Canine oral papilloma virus, Lane M: 100 bp DNA ladder, Lane 1: Sample-E6 gene of COPV, Lane 2: Sample-E7 gene of COPV, Lane 3: Sample-L1 gene of COPV

The partial sequences of E6, E7 and L1 gene fragments of COPV were submitted to GenBank and the accession numbers MH311871, MH311872 and MH311873 were obtained respectively.

A multiple sequence alignment was carried out using the sequences obtained in this study with published COPV sequences retrieved from NCBI. The COPV of OND strain generated in this study was closely clustered with COPV 1 belonging to *Lambdapapillomavirus* genus based on phylogenetic analysis of E6, E7 and L1 sequences. The analysis of sequences generated in the present study showed 100% homology with other COPV *Lambdapapillomavirus* 2 sequences available in the NCBI database by blast search. The phylogenetic tree of E6, E7 and L1 genes of COPV were shown in figures 2, 3 and 4 respectively.

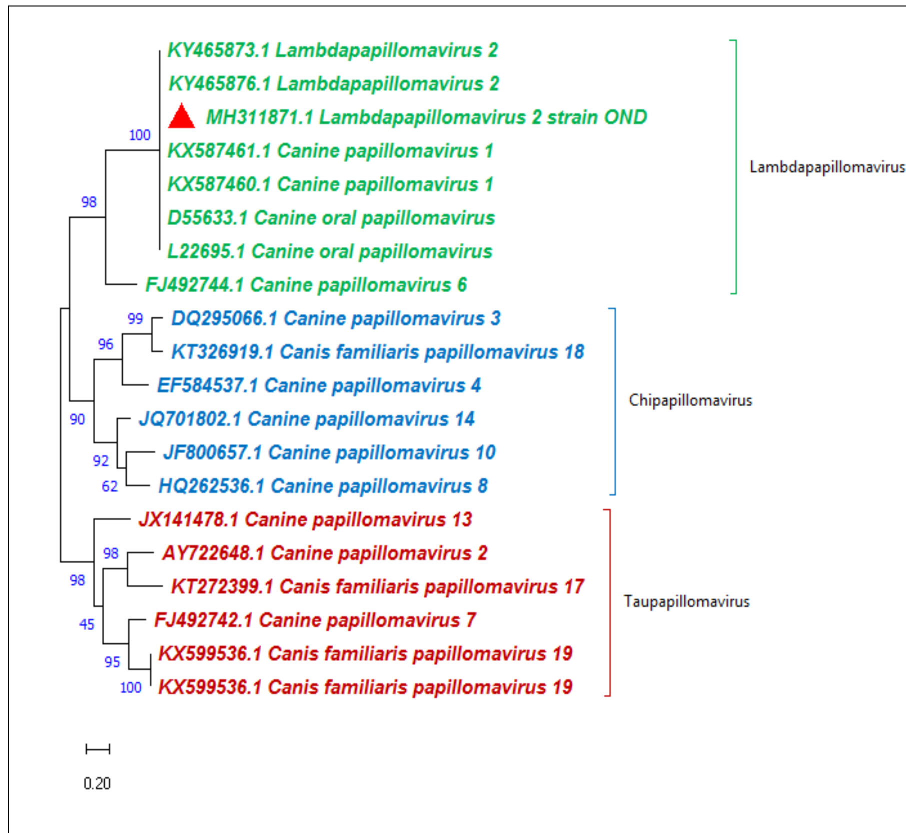

Fig 2: Phylogenetic tree of partial E6 gene of COPV with selected reference sequences. The COPV OND strain in this study is marked in red triangular. The tree was constructed by maximum-likelihood method with 1000 bootstrap replicates using Tamura-Nei model in MEGA 11 software

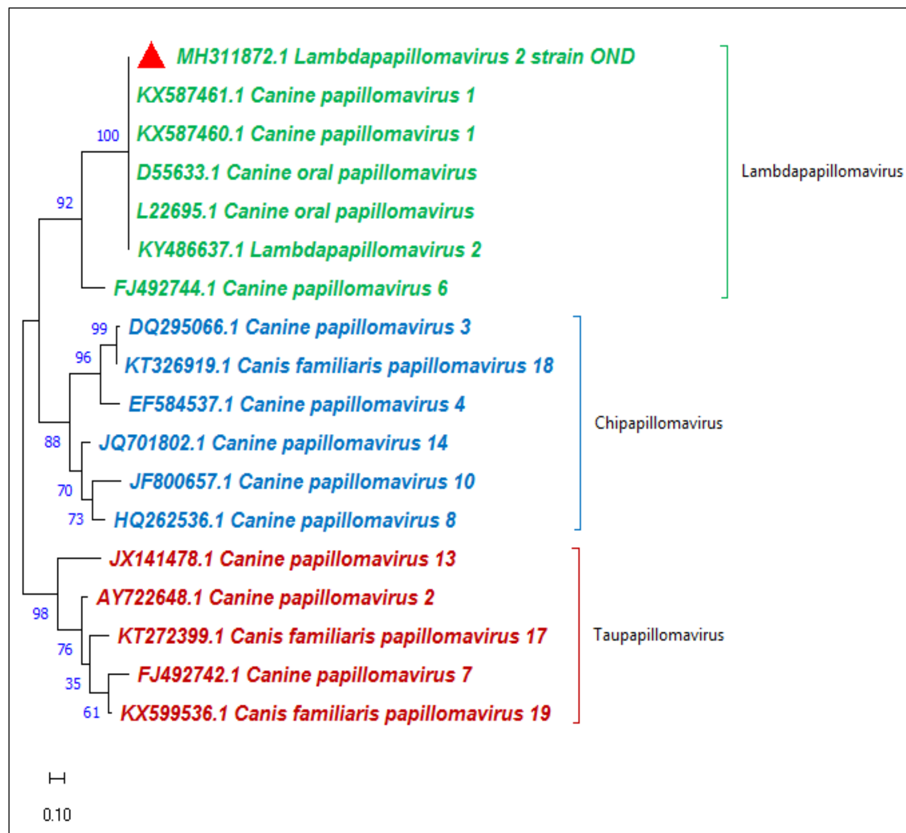

Fig 3: Phylogenetic tree of partial E7 gene of COPV with selected reference sequences. The COPV OND strain in this study is marked in red triangular. The tree was constructed by maximum-likelihood method with 1000 bootstrap replicates using Tamura-Nei model in MEGA 11 software

Fig 4: Phylogenetic tree of partial L1 gene of COPV with selected reference sequences. The COPV OND strain in this study is marked in red triangular. The tree was constructed by maximum-likelihood method with 1000 bootstrap replicates using Tamura-Nei model in MEGA 11 software.

4. Discussion

Papillomaviruses are species-specific and infect the epithelial cells of mucosal or cutaneous sites of mammals, birds and reptiles (Rector and Van Ranst, 2013) [9]. Oral papillomatosis in dogs is caused by CPV1 which is highly contagious and affects young dogs aged below one year. CPV1 causes papillomas as cauliflower-like exophytic warts in the oral mucosa, lips and mucocutaneous junctions which appear 3–4 weeks after infection and last for about 4 weeks before spontaneous regression (Lange and Favrot, 2011; Nicholls *et al*., 2001) [3, 10].

In the present study, PCR was used for the detection of COPV by using gene specific primers targeting the E6, E7 and L1 regions of gene fragments of COPV. The genome of papillomavirus was reported to be circular and approximately 8 kb in size that consists of eight open reading frames which codes early (E1, E2, E4, E5, E6 and E7) and late (L1 and L2) viral proteins (Zheng and Baker, 2006; Bernard *et al*., 2010) [11, 2]. The early E6 and E7 proteins are accountable for cellular proliferation and transformation. The E7 protein is also considered as most efficient cell cycle deregulator. The E6 and E7 oncoproteins were reported to be the inhibitors of p53 and Rb tumor suppressor family respectively (Westrich *et al.,* 2017; Suarez and Trave, 2018) [12-13]. The genome arrangement of canine papillomavirus is similar to other papillomaviruses except for the E5 region which is absent in canine papillomavirus (Nicholls and Stanley, 1999) [14]. The late L1 and L2 proteins are responsible for the formation of viral capsid and package the viral DNA (Doorbar, 2005) [15]. L1 gene is highly conserved and mostly used for the classification of papillomaviruses including canine papillomaviruses (De Villiers *et al*., 2004; Yuan *et al*., 2007) [6, 16]. The sample from the dog gave positive results for COPV which specifically amplified approximately 350 bp, 204 bp and 261 bp lengths for the E6, E7 and L1 gene fragments of COPV respectively. The results in the present study were similar to earlier reports for the detection of COPV (Brandes *et al*., 2009) [8].

Further, the purified amplicons of E6, E7 and L1 of COPV were subjected to sequencing and closely clustered with previously reported *Lambdapapaillomavirus* sequences available in the NCBI database. The partial sequences of E6, E7 and L1 genes of COPV showed 97-100% similarity with the available sequences on the comparison.

5. Conclusion

It is concluded that the phylogenetic analysis of E6, E7 and L1 region of gene fragment confirmed the infection of canine oral warts and the causative agent as COPV. This study describes the incidence of canine oral papilloma infection and discloses the partial E6, E7 and L1 gene sequence information of COPV in the delta region of Tamil Nadu. The identification and molecular characterization are important for effective disease control.

6. Conflict of Interest

Not available

7. Financial Support

Not available

8. References

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