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Electron microscopy and histopathological examination of canine papilomavirus

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ABSTRACT

Diagnosis of canine papillomavirus (CPV) infection by histopathology, transmission and scanning electron microscopy is presented. The study is based on data obtained by examining nonregressing papillomas (warts) from naturally infected dogs with clinical manifestations of CPV infection. Papules on the mouth and lips were common bilaterally in 6 dogs. Confirmatory diagnosis of sick dogs was made by clinical findings, histopathology, transmission and scanning electron microscopy. Histopathological examination of hematoxylin and eosin stained papillomas revealed lymphoplasmocytic cell infiltration and fibrosis, parakeratosis in the dermis, papillary proliferation and intranuclear vacuole degeneration in the stratum spinosum. Electron microscopy demonstrated viral icosahedral capsid formation and non-enveloped viral structure of CPV. Transmission electron microscopy demonstrated viral particles and virions in the nuclei of infected cells, viral crystal mode formation in the nucleus. Scanning electron microscopy demonstrated virions and virus-like particles budding in the infected tissue. The findings of the study reveal that electron microscopy and histopathology are effective and sensitive methods in the diagnosis of CPV infection. Electron microscopy is the only imaging technique that allows direct visualization of viruses, along with affected tissues and cells, due to its nanometer-scale resolution. This study reveals the intracellular and extracellular viral pathogenesis, viral ultra structure and structural components of CPV. Present findings indicate canine papillomavirus causes canine papillomatosis, inclusion bodies are common in nonregressive infection, papillomavirus induces cytopathic effect and pathogenesis, viral particles are located in the cell and form crystal mode in nuclear space.

Keywords: Canine papillomavirus, scanning electron microcopy, transmission electron microscopy, histopathology, viral particles.

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Introduction

Papillomaviruses are small, non-enveloped viruses with circular double-stranded DNA genome size of 5748 to 8,607 bp. and belongs to Papillomaviridae family. Taxonomically have two subfamilies, in more than 50 genera and 130 species. The virions have a diameter of 52-55 nm and icosahedral capsid assembly (Doorslaer et al., 2018; ICTV, 2018). The virus genome consists of three main regions: the early region and late regions encode proteins E1 to E7 and L1 and L2, respectively, while the non-coding "long control region" regulates viral gene transcription and replication (Bernard et al., 2010; Doorslaer et al., 2018; ICTV, 2018). Papillomaviruses have been isolated from mammals, birds, fish and reptiles to date and are highly species

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specific (Murphy et al., 1999; Ogawa et al., 2004; Sterling et al., 2014; Tan et al., 2012; Tekelioglu et al., 2017). The papillomaviruses has a high tropism to the mucosal and keratinized epithelium. Cutaneous and mucocutaneous infection occurs and has been associated with squamous cell carcinomas. Papillomaviruses have been classified into different genotypes according to the degree of sequence variation (Bernard et al., 2010; Lange and Favrout, 2011; Doorslaer et al., 2018; ICTV, 2018).

CPV often causes benign skin tumors in dogs called warts, which are typically small, cauliflower or solid-shaped and rough and irregular growths. Warts contain large amounts of infectious virus that are relatively stable in the environment. Transmission between animals occurs through direct or indirect contact with contaminated fence posts or halters. Tattooing or tagging with equipment containing the virus is another common source of infection (Lange et al., 2011; Matheus et al., 2011; Murphy et al., 1999; Nichols et al., 1999; Tekelioglu et al., 2017). CPV warts usually appear on the lips, mouth and skin of dogs, and less frequently on the eyelids and even on the surface of the eyes or between the toes. Warts are usually found in groups rather than single growths (Matheus et al., 2011). The duration of the infection is very variable, from one month to one year, and recurrence is possible (Lange et al., 2011; Nichols et al., 1999; Tekelioglu et al., 2017).

The aim of the present study was to confirm the diagnosis of the viral disease by imaging the virions and virus-like particles of CPV. The use of electron microscopy and histopathology are among the recommended gold standards for diagnosis and were used in this study in accordance with the catch-all principle (Martins et al., 2008; Goldsmith and Miller, 2009, Platter and Hosttetter, 2009; Gentile and Gelderblom, 2014; Roingeard et al., 2018; Gelderblom and Madeley, 2018; Cheng et al, 2020). This study is designed to reveal the intracellular and extracellular viral pathogenesis, viral ultra structure and structural components of CPV.

Materials and Method

Sampling: Aggressive nonregressing papillomatosis of naturally infected six dogs presenting clinical symptoms of CPV in 2017-2021 were investigated with the consent of their owners. These are the dogs referred to Çukurova University Ceyhan Veterinary Faculty Virology Department for consultation. Papillomas were excised surgically and removed forhistopathological diagnosis and further investigations. After excision of papillomas, the sick dogs were provided with the good veterinary practice and care.

Histopathology: Biopsy samples from papillomas were immediately fixed in 10% neutral buffered formalin for histopathological examination. Definitive diagnosis was done at a private veterinary pathology diagnostic laboratory. Following the fixation process, the samples were embedded in paraffin blocks and 4-5 µm sections were cut, stained with hematoxylin and eosin (HE), and examined with a light microscope and their images were recorded.

Electron Microscopy: Scanning and transmission electron microscopy were done byfixation of excised papillomas in 5% glutaraldehyde in Millonig's phosphate buffer at pH 7,4 followed by post fixation step with 1% osmium tetroxide in the same phosphate buffer at 4 °C according to the procedures described by the Electron Microscopy unit of the Çukurova University Central Research Laboratory and Faculty of Medicine Department of Histology and Embryology. Following preparation, specimens were examined and photographed under scanning electron microscopy (SEM FEI, Quanta 650 Field Emission SEM, USA), at 20KV. Sections were also examined and photographed under transmission electron microscopy (JEOL JEM-1400, Japan). The exposed surface was tracked by quadrant, and then photographed at increasing magnification**.**

Results

Papillomas (warts)of different sizes were observed extensively around the mouth, nose and lips in all dogs and were indicated by arrows (Figure 1).Findings were recorded and detailed information about sick dogs is given in Table 1. One of the dogs was 16 years old and the other 5 dogs were younger than 8 months old and two of the sick dogs were female and four were male. The breeds of the dogs were; Cane Corsa (n:1), Çatalburun / Germanpointer mix (n:1) and mixed (n:4), respectively.

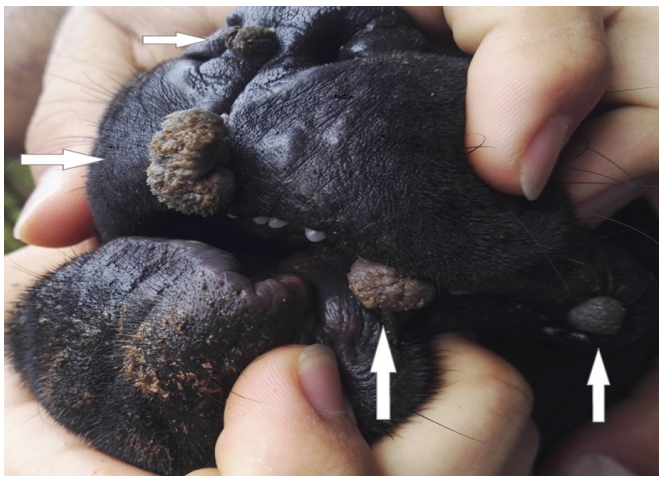

Figure 1. Different sizes of multiple papillomas (warts) at the mouth, nose and lips of the dog (arrows).

Table 1. Data table of sick dogs

Breed	Gender	Age (year)	PapillomLocalisation
Çatalburun/German Pointer mix	Male	16	Mouth + Nose
Mıx	Male	0.6	Mouth + Nose
Mıx	Female	0.5	Mouth
Mıx	Male	0.8	Mouth
Mıx	Female	0.6	Mouth
CaneCorsa	Male	0.5	Mouth + Nose

Macroscopically, gray-brown, moderately firm to hard polypoid growth with skin on its surface was observed. When scanned with serial sections, the incision sections were observed to be gray brown and yellow brown. Histopathological examination of the serial sections of six cases revealed common findings including parakeratotic hyperkeratosis on the surface, increase in keratocytes and papillary proliferations in the squamous epithelium. In the nuclei of keratocyte and spinosum cells, anionucleosis, vesicle formations of varying diameters, marginal chromasia, and two to three nucleolus were observed. Eosinophilic, intranuclear inclusion-like materials were seen in some cells. Disruption of the basement membrane and diffuse lymphplasmocytic cell infiltration was evident in the stroma (Figure 2).

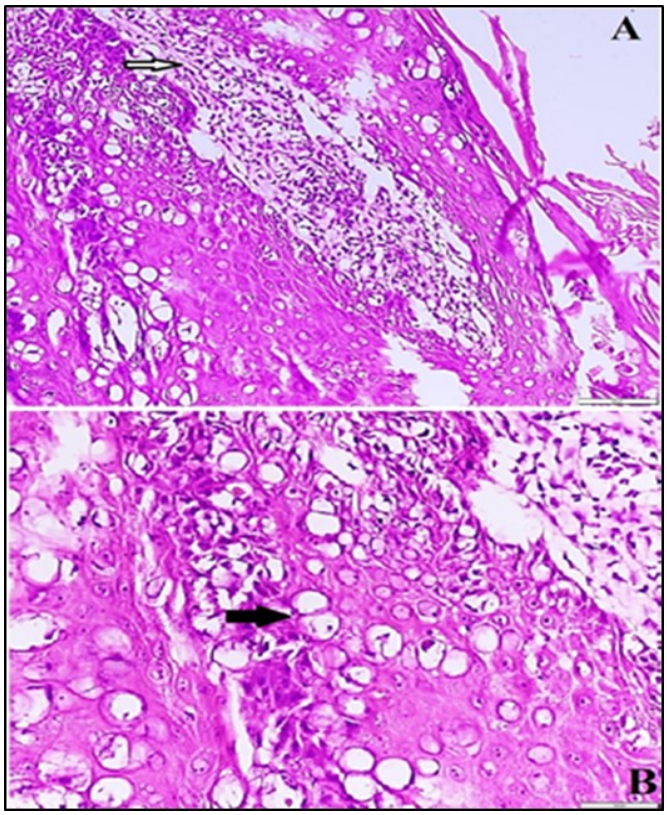

Figure 2. HE stained histopathological images. A; Lymphoplasmacytic infiltrates and fibrosis in dermis (white arrow), B; intranuclear vacuolar degeneration in spinosum cells (black arrow), (bar=100µm).

Scanning electron microscopy (SEM) revealed the damage to mucosal tissues infected by CPV. Characteristic numerous epithelial papillomatous protruding growths of different sizes with diffused exfoliated superficial cells were observed from the inner layers of the infected mucosal tissue. Areas of cellular desquamation were observed more intensely in certain regions, and characteristic papillamatous growths of variable size were observed, and smaller growths of similar nature appeared among larger ones (Figure 3). Due to CPV infection, deterioration was detected in the inner layers of the epidermis, stratum spinosum and stratum basale (Figure 3 a-500X; b-15.000X; c-30.000X).

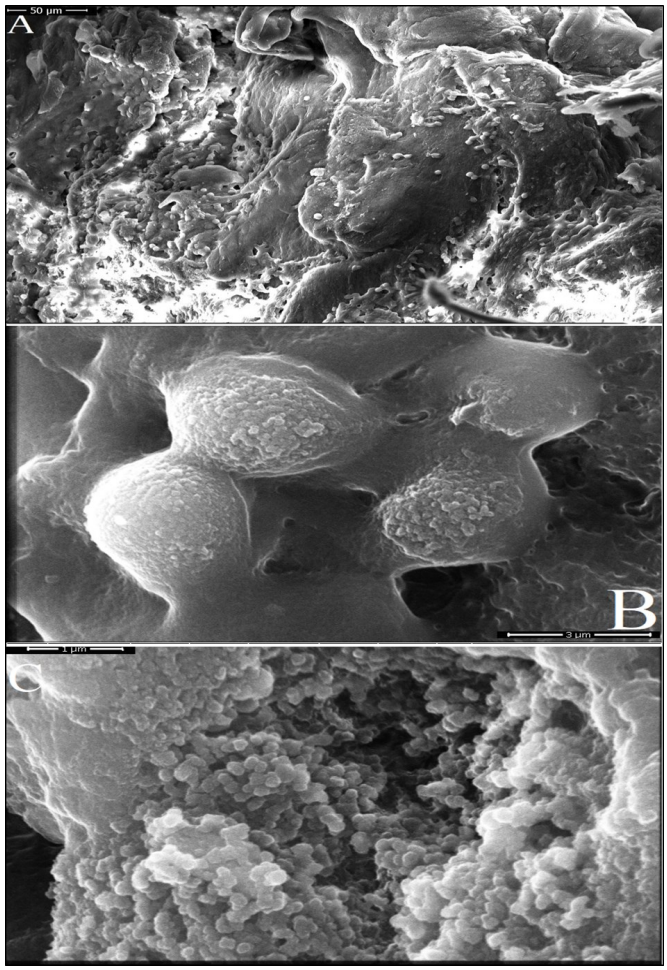

Figure 3. Scanning electron microscopy images of the inner surface of the infected oral mucosa. Observation of disruption of the inner layers of the epidermis, stratum spinosum and stratum basale and protruding growths and scattered exfoliated cells (images and magnification; at 20.00 kV; A; 500X, B; 15.000X, C;30.000X).

Transmission electron microscopy (TEM) revealed the damages to infected cells by CPV. Presence of perinuclear vacuolization was evident in both light and transmission electron microscopy. In CPV-infected cells, nuclei were observed to contain decondensed chromatin and evident nucleoli. Viruses and virus-like particles were observed in the nucleus with the mode of crystal aggregation mode in nuclear space. Various organelles were found in the cytoplasm, especially the mitochondria, which suggests intense cell metabolism. In the examined tissues, it was determined that the spaces between the interdigitated intercellular cells were widened, and it was concluded that this situation had an effect of disrupting the desmosomal structure (Figure 4 a,b,c).

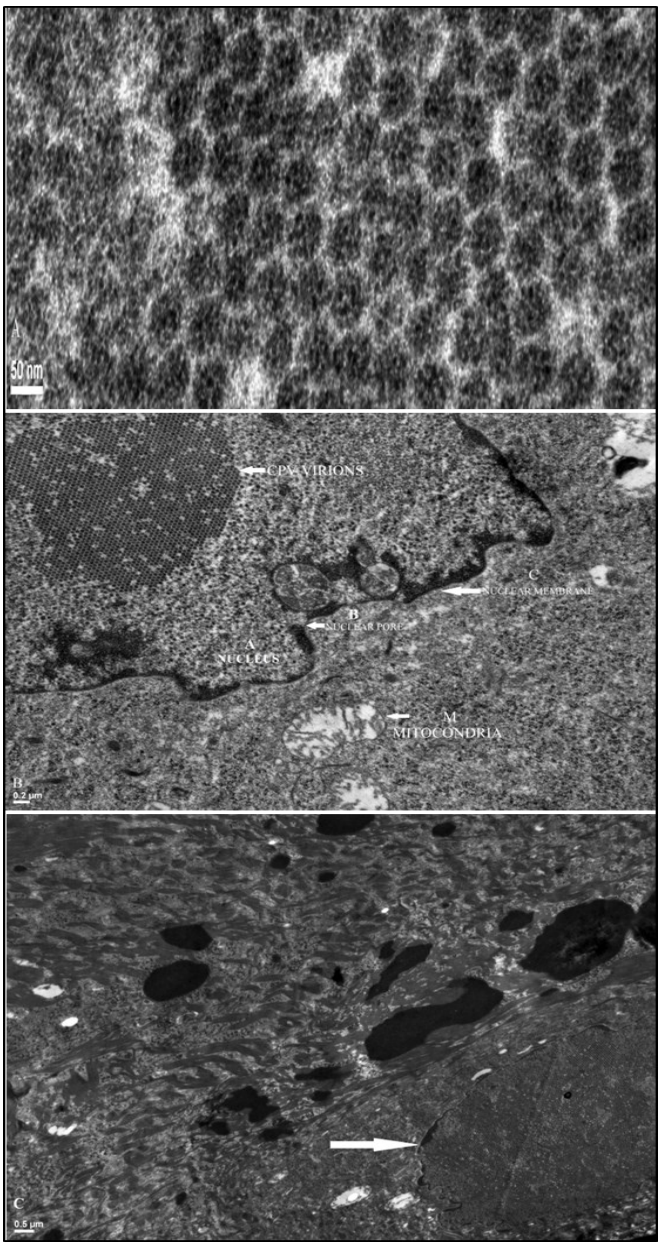

Figure 4. Transmission electron microscopy images of CPV infected cells. A; Aggregation of numerous CPV virions (aprox. 50 nm and icosahedral shaped) as a result of dense clustering within an epithelial cell nucleus. A closely packed array of crystalline virus particles with a packing arrangement. B; Arrows; Nucleus (A), Nuclear Pore (B), Nuclear Membrane (C), CPV virions (CPV); Crystalline formations of virus particles remain in a disintegrated nucleus. Mitocondria (M). C; An intranuclear inclusion body consisting of numerous close-packed aggregates of virus particles with decondensed chromatin.

Discussion

CPV is the etiological agent of canine papillomatosis, which is characterized by benign neoplasms, commonly known as warts, localized mostly in the oro-nasal region, which can spread to the tongue, pharynx and skin (Figure 1). Moreover, in dogs, several canine papillomaviruses (CPVs) have been identified in malignant lesions and are suggested as one of the risk factors for the development of squamous cell carcinomas (SCCs)recently (Gürgen et al, 2020; Chang et al, 2021). The host range of papillomaviruses are very narrow and highly species specific.

Five of the dogs examined in the study were younger than 8 months, and one dog was 16 years old.CPV infection is more common in young dogs and no breed or gender related susceptibility has been previously reported by most (Christian and Claude, 2011; Lange et al, 2011; Sykes and Luff, 2014) accept Bianchi et al. (2012). Interestingly, five dogs were mixed breed and younger than 8 months old in the present study (Table1). These are the dogs referred to Çukurova University Ceyhan Veterinary Faculty Virology Department for consultation.

Results of the histopathological differential diagnosis examination, characteristic superficial vacuolated (koilocytic) keratinocytes specific to the pathogenesis of CPV infection were observed as a typical finding in productive papillomavirus infections. Histopathological examination revealed hyperpigmentation and proliferation in the ephitelia, eosinophilic intra-nuclear inclusion bodies in the stratum granulosum, invasion in stroma, keratinization and polymorphism in the nucleus, as described previously by others (Nicholis et al, 1999; Martins et al, 2008; Platter and Holstetter, 2009; Tekelioglu et al,2017; Chang et al, 2020).In contrast with the findings of Bianchi et al. (2012), intranuclear inclusion bodies were observed in all cases.

It was demonstrated in this study that CPV has a high affinity for the nuclei of epithelial cells and the method of identifying the virus in these tissues by both light and scanning and transmission electron microscopy. The virus-like particles observed in infected cells were found morphologically similar to the findings caused by canine papilloma viruses such as dimension ranging as ~45- 50 nm, replication in the nucleus with the formation of nuclear inclusion bodies

and crystal aggregation mode in nuclear space. As reported by others (Watrach 1969, Nicholis et al, 1999; Martins et al, 2008;Platter and Holstetter, 2009), papillomaviruses have a tendency to collect in the host cell in a crystal structure, which is consistent with the CPV findings of this study (Figure 4).

Some differences can be observed during imaging in the estimated sizes of the same viruses in electron microscopy, these differences may be due to the effects of fixation and electron density differences of viral components. This phenomenon was previously described by Watrach (1969). Clinical, histopathological, and both SEM and TEM electron microscopy findings of this study indicate that the etiologic agent is canine papillomavirus. Similar to our findings explaining the various imaging differences of the same viruses in the cell by electron microscopy, it was also published by other researchers in previous years (Watrach, 1969; Nicholls and Stanley, 1999; Martins et al, 2008).

In the literature, few canine papillomavirus studies based on scanning electron microscopy were reported so far. Nicholls and Stanley, (1999) and Martins et al., (2008) reported epithelial protrusions and exfoliation of cells in affected tissues, similar to the findings of this study.

In the TEM findings, it was observed that the nuclei of the infected cells containing the CPV virion and virus like particles contained decondensed chromatin and the nucleoli were prominent. Various organelles were seen in the cytoplasm and especially the presence of mitochondria, suggesting intense cell metabolism. Additionally, CPVs in crystalline form were observed in the nuclei of the examined infected cells. The formation of the crystal structure requires the viral particles to be similar in shape and structure, the surface arrangement to be equivalent, and the purity of the aggregating particles. The pathological activity, structural and capsid properties of the virus were investigated by examining the crystallization structure formed by the CPV particles in the core cavity. The structural findings regarding the pathogenesis and crystal mode formation occurring in the infected cell and its nucleus are similar to the reports published in previous years by others (Watrach, 1969; Nicholls and Stanley, 1999; Narama et al., 2005; Martins et al, 2008; Platter and Hosttetter, 2009; Wang et al, 2010).

Upon the literature, deeper structures such as the basement membrane and the adjacent chorion are preserved in papillomavirus infections and are not affected by the infection. In this study, it was observed that an aggressive nonregressive pathogenesis occurred in the tissues examined and the basement membrane was also affected by the infection. It has been stated that similar aggressive pathogenesis may occur in infections caused by HPV 11, 16 and 18 subtypes, which are associated with airway malignancies, especially in humans (Martins et al, 2008;Chang et al, 2021).Particular types of papillomaviruses are associated with squamous cell carcinomas in dogs and cancer of cervix, anus and pharynx in humans (Muphy et al, 1999; Martins et al, 2008; Wang et al, 2010; Chang et al, 2020; Gürgen et al, 2021).The relationship of canine papillomaviruses with squamous cell carcinoma and other malignancies in the field of veterinary medicine is still not fully elucidated and studies are ongoing.

Present findings indicate papillomavirus causes canine papillomatosis, inclusion bodies are common in nonregressive CPV infection, papillomavirus induces cytopathic effect and pathogenesis, viral particles are located in the cell and form crystal mode in nuclear space.

Conclusion

The present study reveals the intracellular and extracellular viral pathogenesis, viral ultra structure and structural components of CPV. TEM and SEM electron microscopy provides an immediate overview of the virus replication, true state, distinctive amount and shape of the CPV in a detailed examination of the pathogenesis of current infection. Histopathologically, characteristic findings for CPV were determined and a confirmatory diagnosis was provided with clinical findings and electron microscopy.

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