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Ovine Papillomaviruses: diversity, pathogenicity, and evolution

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Abstract

The family *Papillomaviridae* includes a plethora of viral species infecting virtually all vertebrates excluding amphibians, with astonishing impact on human and animal health. Although more than 250 species have been described in humans, the total number of papillomaviruses (PVs) discovered in animals does not reach up to this number. In animals, PV infections are mostly asymptomatic or can cause variable clinical conditions ranging from self-limiting papillomas and other cutaneous and mucosal benign lesions to cancer. Most of animal PV types have been discovered in cattle, dogs, horses, and cats with other farm host species remaining overlooked. In particular, the number of PV types so far identified in sheep is limited. This paper comprehensively reviews ovine PVs features, including viral taxonomy and evolution; genome organization; viral tropism and pathogenesis; macroscopical features and histopathological patterns, as well as available diagnostics tools. Data are critically presented and discussed in terms of impact on veterinary and public health. The development of future dedicated research is also discussed.

1. Introduction

Papillomaviruses (PVs, *Papillomaviridae*) are small, non-enveloped viruses characterized by a small circular double-stranded DNA genome of about 8 kb in size (Van Doorslaer, 2013a). They

are generally considered species-specific and infect epithelial cells of a wide variety of animal hosts, ranging from fish to birds, reptiles, humans, and other mammals (Van Doorslaer, 2022).

Since their first discovery in the Last Century, PVs have been increasingly studied for their potential role in the development of proliferative lesions of animals and humans, and their association with cutaneous warts and papillomas (Shope and Weston Hurst, n.d.; Orth et al., 1978).

In humans, PVs are responsible for the development of benign papillomas as well as cutaneous, anogenital, and oropharyngeal cancers, by persistently infecting basal keratinocytes in cutaneous and mucosal sites and adapting viral replication to cell differentiation toward upper layers (Nelson and Mirabello, 2023). Viral activity dualistically interferes with cell cycle, promoting on the one hand the proliferation of superficial epithelial layers, and on the other hand inactivating cell-cycle checkpoints in the basal epithelial cells (McBride, 2017). Indeed, PVs tumorigenic potential mostly relies on the activity of their E6 and E7 oncogenes, which cause degradation of the tumour suppressors p53 and pRb, respectively (Nelson and Mirabello, 2023). However, growing evidence points out that oncogenic transformation induced by PVs should be considered a by-product of viral-host molecular interaction, and that with the exception of high-risk human viruses, PV-mediated cellular transformation always requires the intervention of an initial stress factor, such as UV light or mutagenic agents (Hasche et al., 2017).

A number of PV species have been detected in the skin and mucosa of domestic and wild animals in association with benign and malignant tumours, such as the canine oral papillomatosis, the equine sarcoid, and the urinary bladder cancer in cattle (Lange et al., 2011; Zhu et al., 2013; Lecis et al., 2014; Da Silva et al., 2016; Roperto et al., 2016, 2018; Munday et al., 2017; Alcântara et al., 2021; Porcellato et al., 2021). So far, especially livestock and companion host species have been investigated for the presence of PV and, according to PAVE (https://pave.niaid.nih.gov/explore/reference_genomes/animal_genomes), 44 PV types have been detected in cattle, 24 in dogs, 10 in horses, and 7 in cats. Despite their relevance as globally farmed livestock animals, sheep are poorly investigated as PV hosts. Notably, cutaneous proliferative lesions

suggestive of PV infection are commonly reported by farmers, especially in tropical areas (A.F. Ahmed and Hassanein, 2012; Tmumen et al., 2016).Recent literature reports insights on *Ovis aries* papillomaviruses (OaPVs), and their oncogenic potential in sheep and other domestic species. (Alberti et al., 2010; Tore et al., 2017).Focus of this review is to frame the current knowledge on ovine papillomaviruses by presenting and discussing their main biological, pathological, and evolutionary features, and the histopathological patterns of associated lesions. Also, main diagnostic tools employed in ovine papillomavirus diagnosis, as well as updated information on their epidemiology and interspecific infection potential are reviewed.

2. Diversity of ovine papillomaviruses

2.1. Taxonomy of ovine PVs

PVs are classified in genera, species, types, and variants based on their L1 gene sequence(Bernard et al., 2010). A genus comprises species sharing >60% nucleotide sequence identity in the L1 ORF. Each genus is identified by a letter of the Greek alphabet and, as the number of the genera overcame the number of the letters, the prefix “dyo”, which means “for the second time”, is used to name new genera. PVs belonging to the same species share between 71% and 89% nucleotide L1identity whereas a new PV type can be designated when L1 differs >10% from the closest type. In case of a sequence difference <2% a variant can be defined.

According to this classification, 4 types of OaPVs have been identified (Table 1), namely OaPV1, OaPV2, (Karlis et al., unpublished) and OaPV4 (Tore et al., 2017), belonging to the *Delta* genus, and OaPV3 assigned to the *Dyokappa* genus (Alberti et al., 2010).

.**Table 1**: Types of ovine PVs and related isolation source, classification, and references

Type	**Species**	**Genus**	**Source**	**Reference**	**Genbank**
OaPV1	Deltapapillomavirus 3	*Delta*	Fibropapilloma	Karlis *et al*., 1997 (unpublished)	U83594.1

OaPV2	Deltapapillomavirus 3	*Delta*	Fibropapilloma	Karlis *et al*., 1997 (unpublished); Munday et al., 2023	U83595
OaPV3	Dyokappapapillomavirus 1	*Dyokappa*	Squamous cell carcinoma	Alberti *et al*., 2010	FJ796965
OaPV4	Deltapapillomavirus 3	*Delta*	Fibropapilloma	Tore *et al*., 2017	KX954121

2.2. Genomic features

(Alberti et al., 2010; Tore et al., 2017)..

Ovine deltapapillomaviruses seem associated with benign fibropapillomas and were never detected in either pre-cancerous or cancer lesions while OaPV3 was detected in squamous cell carcinomas (SCC) cases and in normal skin (Karlis et al., unpublished, Bariloche et al., 2000a; Alberti et al., 2010; Tore et al., 2017).

Ovine PVs genome size slightly differs according to the genus, with OaPV1, OaPV2, and OaPV4 having a longer genome (7761 bp, 7758 bp, and 7758 bp, respectively) than OaPV3 (7344 bp). The GC content is similar, spanning from 45.7% in OaPV1 to 48.8% in OaPV3. The essential viral core organization (E6, E7, E1, E2, L2, and L1) is shared by all ovine PVs genomes (Fig. 1). The long control region LCR, located between the L1 stop codon and the E6 start codon, varies in size among the different ovine PVs (734 bp, 737 bp, 635 bp, and 731 bp in OaPV1, OaPV2, OaPV3, and in OaPV4, respectively). The ovine PVs LCRs contain several binding sites. The E1 binding site motive (A(A/T)GATTGTTGTTAACAAT, E1BS) identified in the LCR of ovine deltapapillomaviruses shows a conserved T at the position 2 in all the 3 members. On the contrary, it was not possible to identify a canonical E1BS in the OaPV3 LCR. The number of E2 binding sites

(E2BS) [ACC-N6-GGT] varies among species (7 in OaPV1, 6 in OaPV2 and OaPV3, and 8 in OaPV4). In OaPV4, two additional E2BS were found within E6 (nt 810) and L1 (nt 7137).

The E1 ORF encodes for the largest PV protein, an ATP-dependent DNA helicase required for initiation of viral DNA replication. It also contains a cyclin interaction RXL motif which mediates E1 interaction with and phosphorylation by cyclin/Cdk complexes and is required for efficient viral replication *in vivo* (Ma et al., 1999).

A putative E4 ORF can be identified in all the ovine PVs as a result of a mRNA splicing processes, unifying the first few codons of E1 (nt 747-762, nt 745-760, nt 1303-1315, nt 1380–1098 for OaPV1, OaPV2, OaPV3, and OaPV4, respectively) with a downstream ORF in the +1 frame of the E2 ORF (nt 3171-3460, nt 3175-3458, nt 3676-4013, nt 3802–4413for OaPV1, OaPV2, OaPV3, and OaPV4, respectively). The late region encodes for the capsid proteins L1 and L2. Both L1 (KRRRK) and L2 (KKKRKKKR) contain a C-terminal series of arginine and lysine residues, which are likely to function as a nuclear localization signal. While the essential viral core organization (E1, E2, L1, and L2) is basically similar in all the ovine PVs, some differences can be detected in the putative oncogenes. An E5 ORF was identified in OaPV1, OaPV2, and OaPV4, as seen in other artiodactyl PVs of the same genus, but not in the OaPV3 genome. The conserved retinoblastoma tumour suppressor binding sequence motif (pRB-binding domain, LXCXE) and the zinc-binding motifs are absent in OaPV1, OaPV2, and OaPV4 E7; interestingly, a putative pRB-binding domain was identified in the E6 ORF. Conversely, in OaPV3 a pRB-binding domain was identified in the E7 ORF, as the zinc-binding motifs (Alberti et al., 2010; Tore et al., 2017). Notably, the presence of an E5 ORF and the lack of a pRB binding domain in E7 have been related to the development of fibropapillomas (Narechania et al., 2004). E6 conserved zinc-binding domains (CXXC-X(28-30)-CXXC) are present in all 4 ovine PVs.

2.3. Phylogenetic and evolutionary features

For a long time, papillomavirus - host coevolution was dated back to more than 300 Million years (Myr), believing their range of infection was restricted to mammals, birds, and reptiles. After the recent identification of *Sparus aurata papillomavirus 1* (SaPV1) in gilthead sea bream *(Sparus aurata*), the first recognized papillomavirus in a fish species, the path of papillomavirus adaptation to its host has been backdated to 120 Myr earlier (López-Bueno et al., 2016).

Papillomaviruses are considered highly host-specific viruses with a slow evolutionary rate, features that would argue in favour of a strict co-evolutionary path with their hosts. However, existing incongruences in papillomavirus distribution on the phylogenetic tree suggest that other driving factors, such as adaptation to ecological niches or cross-infections, could have interfered with the linearity of this process (Van Doorslaer, 2013b). This is the case of ovine papillomaviruses, which are divided between two different genera, *Delta* and *Dyokappa* (Fig. 2). Examination of nucleotide and amino acid sequence alignments indeed confirmed the polyphyletic nature of OaPVs by showing the low shared identity of OaPV3 with other OaPVs of the *Delta* genus (Alberti et al., 2010; Tore et al., 2017). OaPV3, the more ancient virus among OaPVs, is thought to have diverged from *Rupicapra rupicapra papillomavirus* (RrPV), with whom shares the *Dyokappa* genus, roughly around the same time as the divergence between the *Ovis* and *Caprinae* genera, around 3.73 (± 1.7) Myr ago (Ropiquet and Hassanin, 2005; Bunch et al., 2006; Tore et al., 2017). Besides, among OaPVs of the *Delta* genus (all belonging to the Delta 3 species), OaPV1 and OaPV4 remained closely interrelated (divergence time at the closer node 0.61 ± 0.24 MYR), whereas OaPV2 slightly diverged from the formers earlier in time (divergence time at the closer node 1.5 ± 0.65 Myr), probably in correspondence of the divergence of the Urial sheep *O. vignei* from the mouflon *O. orientalis,* about 1.3 Myr (Rezaei et al., 2010; Tore et al., 2017).

3. Ovine PVs-host interactions

3.1. Tissue tropism and tumorigenic potential

The causal link between papillomavirus infection and cutaneous neoplastic lesions was demonstrated in sheep for the first time by Gibbs and colleagues in 1975, through the detection of papillomavirus particles in cutaneous cells of sheep fibropapillomas via electron microscopy (Gibbs et al., 1975). In this study, authors proved the transmissibility of this infection and the consequent occurrence of fibropapillomas in sheep, but not in recipient cattle and goats. This latter finding suggested that a new viral agent, specific for the ovine species, could have been responsible for fibropapilloma development in sheep. However, isolation and characterization of two complete ovine papillomavirus genomes (namely OaPV1 and OaPV2) were achieved for the first time only in the late '90s, from an Australian merino sheep flock (Karlis et al., 1997 unpublished). The third of OaPVs, Ovine papillomavirus3, was detected by Alberti and colleagues (2010) associated with squamous cell carcinomas exclusively within neoplastic and normal epithelial cells by *in situ* hybridization (ISH), whereas OaPV4, recently identified by Tore *et al.* (2017), was detected in both epithelial cells and fibroblasts of cutaneous fibropapilloma (Alberti et al., 2010; Tore et al., 2017).

The variety of lesions associated with ovine papillomaviruses reflects the tropism for different histotypes and OaPVs' specific tumorigenic potential. Ovine *Delta* were described as associated with the development of fibropapillomas, along with other ruminant *Artiodactyla* PVs (Narechania et al., 2004; Munday et al., 2023a). The tropism for fibroblasts is indeed a distinctive feature of most viruses of this genus, differently from most PVs that usually target epithelial cells (Narechania et al., 2004). This peculiarity probably reflects a different pathogenetic process which could have diverged from the original epithelial tropism during early evolutionary phases (Jarrett et al., 1984). More specifically, viruses of the *Delta* genus have a putative pRB binding domain in E6, commonly lack retinoblastoma protein (pRB) binding domain in E7 ORF, and possess a highly conserved E5 gene, a genome integration dating back between 65 and 23 Myr ago (García-Vallvé et al., 2005). Intriguingly,

these two latter features have been associated with the development of fibropapillomas (Narechania et al., 2004; Tore et al., 2017). It is known that BPVs of the *Delta* genus exert their transforming activity by E5 gene interaction with tyrosine kinase growth factor receptors, specifically EGF and PDGF receptors (Münger and Howley, 2002). Additionally, the presence of 4 amino acids in the E5 protein (glutamine at position 26, aspartic acid at position 42, and two cysteines at positions 46 and 48), necessary for the biological activity of BPV1 in cultured fibroblast, has also been identified in OaPV4 E5 protein (Tore et al., 2017). As members of the *Delta* genus, it is thus conceivable that OaPV1, OaPV2, and OaPV4 could trigger oncogenic transformation in fibroblast by activating the tyrosine kinase receptors pathway.

OaPV3 (*Dyokappa*) has been detected only in epithelial cells of the normal epithelium and in squamous neoplastic keratinocytes, suggesting it substantially differs from other OaPVs and shares more similarities with epitheliotropic viruses of other genera found in association with malignant epithelial neoplasms (Alberti et al, 2010, Tore et al., 2017). Its oncogenic activity seems to rely on the calpain-mediated degradation of ovine pRb promoted by E7 activity, which is known to be capable of primary keratinocytes immortalization in sheep and to exert key functions on cell proliferation and viral replication (Scarth et al., 2021). This hypothesis has been recently reinforced by the detection of high levels of activated calpain-1 and hyperphosphorylated pRB in bladder tumours of cattle bearing OaPVs infection (De Falco et al., 2023).

However, although PVs have been found in association with malignant tumours, they aren't thought to act as promoters of neoplastic transformation alone, but rather to act jointly with other predisposing factors, such as ultraviolet radiation (UVR) or immunosuppression induced by bracken fern ingestion (Roperto et al., 2016; Fania et al., 2021). The synergistic role of PVs transforming properties, combined with mutagenic effects of prolonged UV radiation, has been supposed by many authors, further corroborated by the detection of increased expression of human papillomavirus oncogenes consequent to p53 activation by UVR (Purdie et al., 1999). This theory has been empirically supposed also for OaPVs-induced cutaneous tumorigenesis in ovine, since first reports

on ovine papillomas and SCCs, after observation of tumours localization in poor-pigmented, sun-exposed skin, such as the head, udder, limb, and perineal and genital area (especially in dock-tailed sheep) jointly with the detection of viral particles in precancerous and cancerous lesions (Gibbs et al., 1975; Vanselow and Spradbrow, 1983; Trenfield et al., 1990; Tilbrook et al., 1992). The detection of a high prevalence of OaPVs DNA in the blood of a heterogeneous population of apparently healthy sheep further strengthened this assumption (De Falco et al., 2021b).

3.2. Macroscopical and microscopical features of associated lesions

Sheep PVs have been reported in association with both hyperplastic lesions and benign and malignant skin tumours (Fig 3), such as cutaneous horns, papillomas, fibropapillomas, and squamous cell carcinomas (Gibbs et al., 1975; Vanselow and Spradbrow, 1983; Trenfield et al., 1990; Tilbrook et al., 1992; Bariloche et al., 2000b; Alberti et al., 2010; Tore et al., 2017; Munday et al., 2023) and their presence has been supposed in association with the development of rumen fibropapillomas (Norval et al., 1985).

Cutaneous horns are small exophytic benign tumour-like lesions, characterized by a markedly hyperplastic epidermis covered by multiple layers of compact keratin (Gibbs et al., 1975; Trenfield et al., 1990; Ginn et al., 2007). These lesions have been reported in the haired skin and mucosa in association with the detection of papillomavirus-like DNA sequences belonging to *Delta* genus and closely related to BPVs, in the early ‘90s (Trenfield et al., 1990).

Papillomas are benign exophytic epithelial neoplasms, found on the haired skin and mucosa. Most reported sites of papillomas in sheep are the head (muzzle, ears, eyelid, lips), udder, and limbs, but they have also been frequently described on the perineal and vulvar area (Trenfield et al., 1990; Bariloche et al., 2000a). At histology, they are characterized by epidermal hyperplastic papillae supported by a thin stalk of fibrovascular stroma, with finger-like epithelial structures that project into the underlying dermis (rete peg). The spinous layer is hyperplastic, and keratinocytes have pale eosinophilic cytoplasm (viral cytopathic effect). Moreover, enlarged keratinocytes with cytoplasmic

clear halos (koilocytes) can be observed (Goldschmidt et al., 1998; Ginn et al., 2007). Papillomavirus has been detected in epidermal keratinocytes of the stratum granulosum (Bariloche et al., 2000a).

Fibropapillomas macroscopically appear as well-defined nodules, plaques, or exophytic lesions in the haired skin (Fig 3). At histological examination, they are characterized by the proliferation of dermal plump fibroblasts, arranged in interlacing bundles, with oval and vesicular nuclei, and prominent nucleoli. The epidermis is hyperplastic and hyperkeratotic, organized in down-growing rete ridges with occasional koilocytes in the stratum spinosum (Ginn et al., 2007). Papillomavirus has been detected in both epithelial cells and proliferating fibroblasts of fibropapillomas (Tore et al., 2017) (Fig 3).

Cutaneous squamous cell carcinomas (cSCC) related to papillomavirus infection in sheep are often described as cauliflower-like exophytic masses of variable size, often ulcerated, that commonly affect the pinnae, eye, eyelids, and udder and to lesser extent muzzle, lower lip, vulva, and perineum (Mendez et al., 1997; Ahmed and Hassanein, 2012; Abo-Aziza et al., 2017) (Fig 3). At histological examination, they are characterized by a proliferation of cuboidal to polyhedral epithelial cells arranged in cords, trabeculae, or islands. Depending on the grade of differentiation, small islands of squamous cells associated with central eosinophilic keratin (keratin pearls) could be present (Fig 3). Neoplastic keratinocytes show moderate to severe anisocytosis, anisokaryosis, and a variable number of atypical mitoses (Fava et al., 2001.; Vitiello et al., 2017). Ovine papillomavirus has been detected in neoplastic epithelial cells (Vanselow and Spradbrow, 1983; Alberti et al., 2010; Vitiello et al., 2017, 2021).

4. Epidemiology and diagnosis of ovine PVs

4.1. Epidemiology, coinfections, and cross-transmission

Ovine papillomaviruses have been scarcely reported since their first recognition. It is known that OaPV1 and OaPV2 were first reported in Australia, whereas OaPV3 and OaPV4 were both discovered in the Sardinian Island (Italy) (Karlis et al., 1997, unpublished; Alberti et al., 2010; Tore et al., 2017). Only a recent systematic investigation on OaPVs DNA prevalence among ovine from southern Italy assessed the predominance of OaPV3 and OaPV4 and detected OaPV1 and OaPV2 for the first time in the Mediterranean area (De Falco et al., 2021b). This study detected OaPVs in 76% of cases, half of which were characterized by coinfections caused by two or more OaPVs. The *Papillomaviridae* family, like a few other DNA virus families, comprises some of the most tissue- and host-specific viruses (Bernard et al., 2010; Geoghegan et al., 2017). However, cross-infections in related species are possible and usually induce the development of similar lesions to those of specific hosts(Bernard et al., 2010). Deltapapillomaviruses have been demonstrated as causative agents of tumours in domestic species other than their natural host. Most notable examples in veterinary medicine are the equine and the feline sarcoids, exuberant fibropapillomas diagnosed in horses and cats, caused by bovine papillomavirus type 1, 2, and 13 (BPV1, BPV2, BPV13) and by bovine papillomavirus type 14 (BPV14), respectively (Chambers et al., 2003; Lunardi et al., 2013; Munday et al., 2015). However, it seems that in the case of sarcoids, PV infection in accidental hosts is restricted only to mesenchymal cells and does not involve epithelial cells as other PVs (Martens et al., 2000; Teifke et al., 2003; Munday et al., 2020).

For a long time, the BPVs of the Delta genus were considered the only group of Deltapapillomaviruses capable of infecting non-host species (Munday et al., 2020). However, recent investigations demonstrated that also OaPVs, originally thought to be limited to ovine, have crossed the interspecific barrier (Munday et al., 2020; De Falco et al., 2022, 2023). In a recent work by De Falco and colleagues (2023), the presence of all four known OaPVs DNA has been demonstrated in

epithelial and mesenchymal neoplasms of the urinary bladder of cattle (*Bos taurus*) by droplet digital polymerase chain reaction (ddPCR) (De Falco et al., 2023). More in detail, OaPV2 was the most frequently identified in tumours, both alone and in combination with other OaPVs, whereas OaPV4 was evidenced in only 20% of samples. The detection of viral RNA expression in all samples pointed out that ovine papillomavirus infection in cattle could trigger, combined with other predisposing factors such as bracken fern ingestion, the bladder carcinogenesis process, regardless of the involved tumour histotype (De Falco et al., 2023). Evidence of significant overexpression and phosphorylation of pRb, calpain-1, E2F3, and PDGFβR in the neoplastic bladder of cattle compared to healthy tissue, further suggested that these molecular pathways could be involved in OaPVs-induced carcinogenesis (De Falco et al., 2023).

Recent research on PVs presence in domestic species further clarified the cross-infection potential of OaPVs (Munday et al., 2020). In an investigation on a gingival sarcoid-like mass in a pet pig, OaPV2 DNA sequences were detected, demonstrating its ability to infect phylogenetically distant species among *Artiodactyla*. Besides, the absence of other papillomaviruses and the similarity of OaPV2 to sarcoid-inducing BPVs suggest a causal relation between OaPV2 and gingival neoplasia (Munday et al., 2020).

Cross-infection of OaPVs has also been proved in the blood of healthy cattle sampled in a slaughterhouse, unrelated to tumour presence, by De Falco and colleagues (2022). OaPVs DNA was detected by ddPCR in the blood of 78% of examined bovines, with a higher prevalence of OaPV1 and OaPV2 and to a lesser extent of OaPV3 and OaPV4, as single infection agents or variably combined (De Falco et al., 2022). Of note, transcriptional activity was frequently spotted for all OaPVs through the detection of L1 and E5, E6, and E7 oncogenes transcripts (De Falco et al., 2022). Interesting insights on the potential transmission routes of ovine papillomavirus to other species have also been offered in the recent literature by De Falco and colleagues (2022), which proposed grazing pastures shared with sheep or ingestion of contaminated food as a possible source of the virus, the

latter supported by the detection of OaPVs DNA in grass hay and corn silage from a cattle intensive farm (De Falco et al., 2022).

4.2. Diagnostic tools

Diagnostic tools for the detection of PVs mostly consist of molecular biology techniques, that allow the detection of viral DNA from both liquid and solid matrices, or *in situ* immunodiagnostic methods that allow the localization of specific viral proteins within host tissues (Trenfield et al., 1990; Tilbrook et al., 1992; Gowans Eric J.and Arthur, 1995). Among molecular tools used for detecting the presence of ovine papillomaviruses DNA in blood and tissue samples, multiple-primed rolling circle amplification (RCA) is a useful technique that enables amplification of small DNA fragments under isothermal conditions(Dean et al., 2001; Nelson et al., 2002). For the detection of OaPVs, the L1 gene has been targeted from cutaneous squamous cell carcinomas and fibropapilloma samples (Tore et al., 2017, 2019).

Digital droplet PCR (ddPCR) is a new generation highly sensitive amplification technique that, by partitioning DNA into million droplets, allows accurate quantification of researched molecules (Isaac et al., 2017; De Falco et al., 2021a). In a recent comparative study on the ability of ddPCR and qPCR to detect different OaPVs DNA from ovine blood samples, the former demonstrated 98.4% sensitivity in contrast to 38.1% of qPCR (De Falco et al., 2021b). However, it should be considered that ddPCR is an extremely sensitive technique, and this could decrease the diagnostic significance of findings, since very small amounts of pathogen DNA can be detected in a lesion or in the blood, even in asymptomatic subjects. Few immunodiagnostic tools are available to localize the presence of PV nucleic acids within lesions in formalin-fixed and paraffin-embedded (FFPE) samples. *In situ* hybridization is a useful tool that allows localization of protein of interest in tissues using specifically designed probes (Trenfield et al., 1990; Tilbrook et al., 1992; Gowans Eric J.and Arthur, 1995). Such technique has been used for the detection of OaPV E6 and L1 proteins, respectively expressed in the early and late phases of the PV life cycle, in OaPV3-positive ovine

SCCs and OaPV4-positive fibropapilloma(Alberti et al., 2010; Vitiello et al., 2017; Tore et al., 2019). More specifically, E6 and L1 DNA probes were produced as described by Alberti *et al.* (2010) and employed in both colorimetric and fluorescence ISH (Alberti et al., 2010; Vitiello et al., 2017; Tore et al., 2019). The results of these studies identified OaPV3 L1 and E6 in neoplastic and non-neoplastic keratinocytes of ovine SCCs and E6 in OaPV4 positive fibropapilloma (Alberti et al., 2010; Vitiello et al., 2017; Tore et al., 2019).

Immunohistochemistry (IHC) represents an alternative tool allowing localization of OaPVs viral proteins in tissues. However, this method has some limitations in cases where integration of the viral DNA hampers production of L1 protein. In human papillomavirus-related oropharyngeal cancer, the detection of high-risk HPV is accomplished by a well-established diagnostic protocol, characterized by IHC followed by *in situ* hybridization for confirmation of IHC-positive cases (Henley-Smith et al., 2021). The lack of available commercial tools for OaPVs diagnosis by *in situ* techniques, such as ISH and IHC, is a limiting factor for the assessment of OaPVs presence within neoplastic lesions in sheep. In two recent investigations by Cacciotto *et al*. (2022) and Tore *et al*. (2019), specific antibodies were raised against OaPV3-E6 and OaPV4-E6 oncoproteins and the OaPV3-L1 capsid protein. Their IHC reactivity was tested in sections of fibropapilloma and squamous cell carcinomas, previously assessed to be OaPV3 and OaPV4 positive by PCR. Results evidenced the presence of a strong cytoplasmatic immunoreactivity for OaPV3-L1 and OaPV3-E6 in neoplastic keratinocytes of SCCs, whereas in fibropapillomas OaPV4-E6 was detected in both proliferating fibroblasts and epithelial cells (Tore et al., 2019, Cacciotto et al., 2023).

5. Conclusions

Despite being overlooked and underinvestigated ovine PVs represent a biologically and pathogenetically diverse group of papillomaviruses. Ovine viruses belonging to *Deltapapillomavirus* show a mixed tropism for epithelial and connective cells (fibroblasts) and have been identified in both healthy sheep (OaPV 1-2-4) and in fibropapillomas (OaPV-4). Ovine *Delta* viruses show some degree of interspecies transmission and have been identified in bladder neoplasms in cattle (OaPV1 and OaPV2) and in a sarcoid-like mass in the mouth of a pig (OaPV2).. OaPV3 *(Dyokappa)*, has been instead associated with cutaneous squamous cell carcinomas in sheep, and has also been found in healthy animals. The association of OaPV3 with tumor development is reinforced by the fact that its closest *Dyokappa* relative, *Rupicapra rupicapra* Papillomavirus type 1 (RrPV1), was also isolated from a malignancy (a nasal neoplasia of a chamois). However, although PVs have been found in association with both benign and malignant tumours, they are not thought to act as a unique promoter of neoplastic transformation, but rather they act jointly with other risk factors. Studies dedicated to OaPVs identification in sheep are scarce, and OaPVs have been rarely reported since their first recognition. Considering the great number of PV types discovered in other vertebrate hosts (human, bovine, dog, horse, and cat), further studies are welcomed to investigate the presence of PVs in sheep, worldwide, and to unveil PV diversity of this overlooked yet multifaceted example of viral diversity, pathogenicity, and evolution.

Figure legends

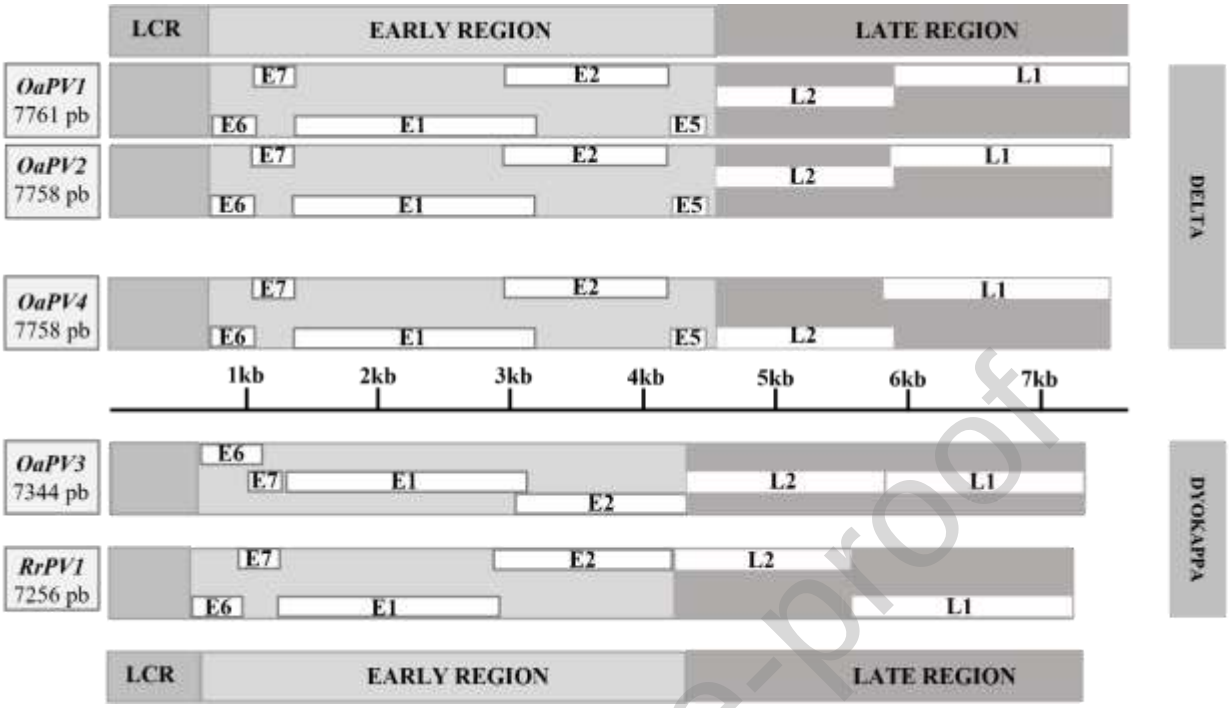

Figure 1. Genome features of OaPVs of *Deltapapillomavirus* and *Dyokappa* genera. *Rupicapra rupicapra type 1 virus* is also shown.

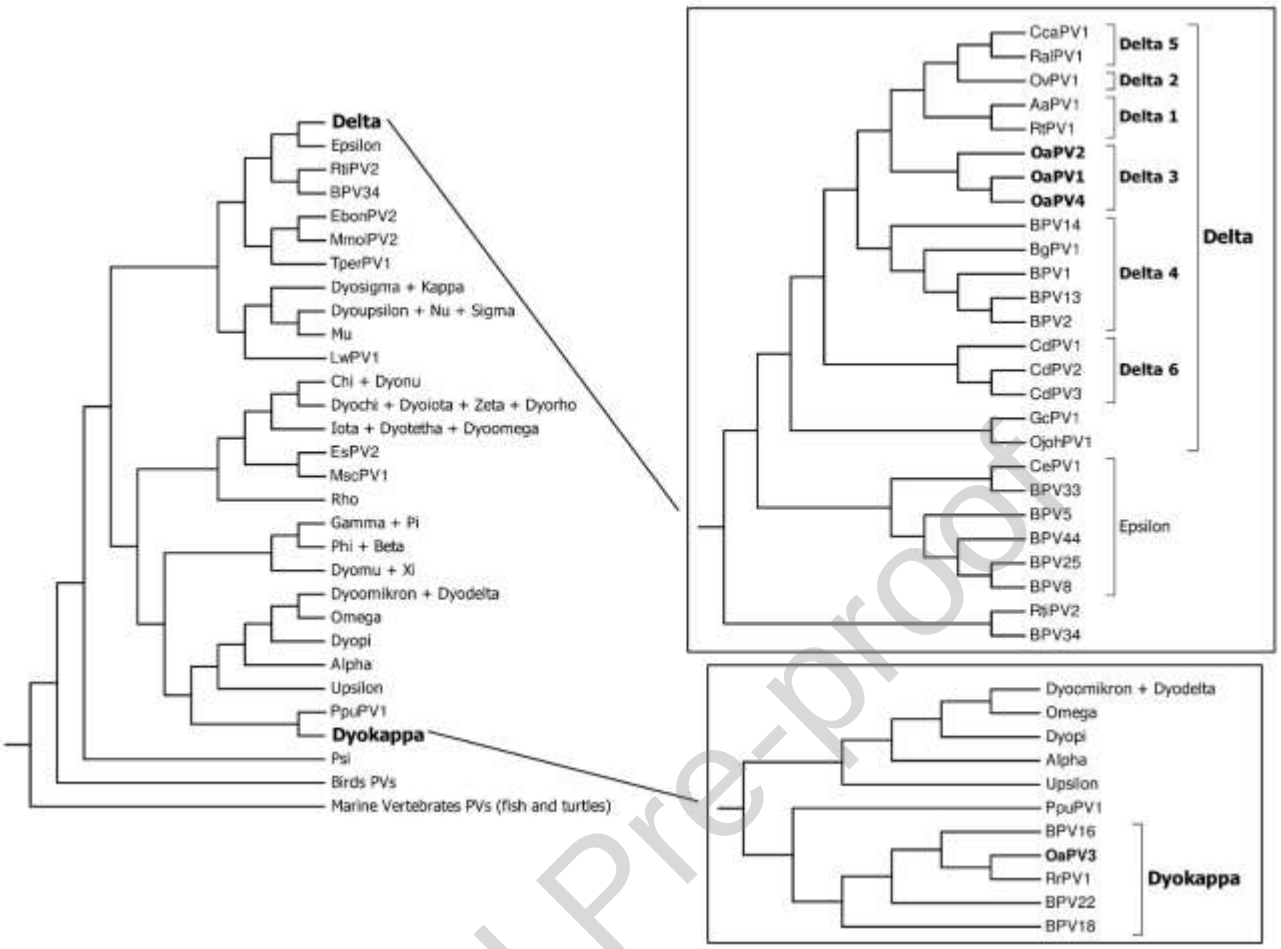

Figure 2. Phylogenetic tree of ovine papillomaviruses (OaPVs). In general, ovine PVs appears to be polyphyletic. The 4 OaPVs (OaPV1 to 4) so far identified fall into 2 different, distant phylogenetic clusters. OaPV1, OaPV2, and OaPV4 form a monophyletic sister group to Delta 1, 2, and 5, while OaPV3 cluster with the oncogenic virus RtPV1 in the Dyokappa group, mostly related to the *Cervidae* virus PpV1.

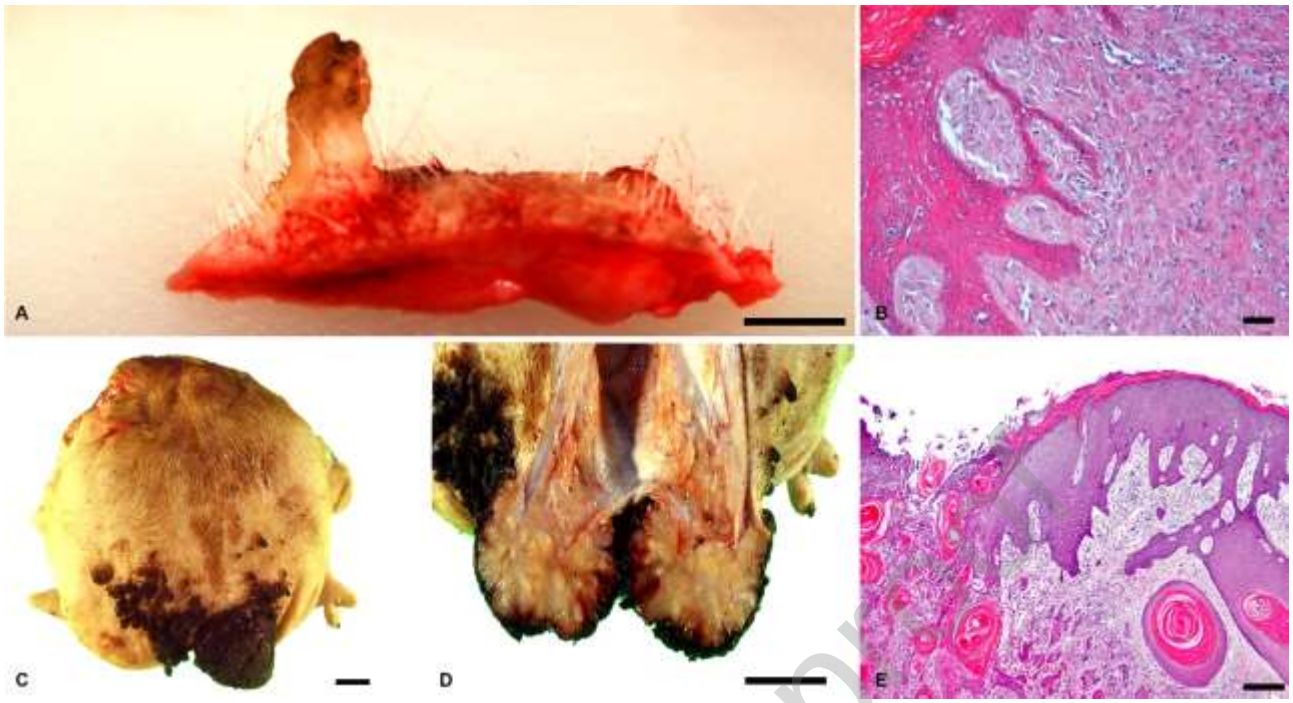

Figure 3. A-E. Ovine skin tumor in the scrotum (A-B) and in the udder (C-D-E). Macroscopical aspect of haired skin fibropapilloma with an exophytic growth pattern (A) (bar = 0.5 cm). Histology of the ovine fibropapilloma: epidermal hyperplasia with rete ridges and fibroblastic proliferation associated with variable amounts of collagenous matrix (B) (HE, bar = 50 µm). Macroscopical aspect of a haired skin squamous cell carcinoma (SCC) with a cauliflower-like aspect associated with multiple neoplastic lesions variable in size (C) (bar = 1 cm) and (D) (bar = 2 cm). Histology of ovine moderately differentiated SCC showing malignant squamous keratinocytes invading the dermis and organized in cords and/or in islands with central eosinophilic keratin (keratin pearls) (E) (HE, bar = 50 µm).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of interests

■ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Ovine PVs are a diverse group of viruses in terms of clinical outcome, taxonomy, evolution and host cell immortalization/transformation
- The 4 ovine PVs are divided into 2 PV genera, each of the 2 associated to different degree of malignancy
- The diversity of ovine PV is still uncovered, and more PV types/species/genera are expected to exist
- OaPV3 can be an animal model of non melanoma skin cancer