Evidence of a novel cross-species transmission by ovine papillomaviruses

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Abstract

Ovine papillomavirus (OaPV) comprises four genotypes; OaPV1, OaPV2, and OaPV4 are fibropapillomaviruses within the genus *Delta*-papillomavirus (*Delta*-PV *)*, whereas OaPV3 is an epitheliotropic virus that belongs to the genus *Dyokappa*-papillomavirus (*Dyokappa*-PV *)*. To date, all of them have been known to infect sheep only. OaPV1, OaPV2, and OaPV4 have been associated with ovine cutaneous and mucosal fibropapillomas, while OaPV3 is a key factor in the squamous cell carcinoma (SCC) pathway of the sheep skin. Peripheral blood mononuclear cell (PBMC) samples obtained from 128 cattle at public slaughterhouses were investigated using droplet digital polymerase chain reaction (ddPCR). ddPCR is a new-generation PCR technique that enables accurate and absolute quantification of target molecules with high sensitivity and specificity. All OaPVs were detected by identification and quantification of nucleic acids using specific fluorescent probes. Of 128 PBMC samples, 100 (˜78%) showed OaPV infections. Further, 42, 35, and 23 PBMC samples showed single, double, and triple OaPV infections, respectively. OaPV1 was responsible for 22 single infections, OaPV2 caused 16 single infections, and OaPV3 and OaPV4 caused two single infections each. OaPV1 and OaPV2 were the most frequent ovine viruses in dual and triple infections. In many PBMC samples, both ovine *Delta*-PV and *Dyokappa*-PV were found to be transcriptionally active, as shown by the detection and quantification of E5 oncogene transcripts for OaPV1, L1 transcripts for OaPV2, E6 and E7 transcripts for OaPV3, and E6 for OaPV4. OaPVs were found in the blood samples from cattle that shared grasslands rich in bracken ferns known to contain immunosuppressant substances. Furthermore, OaPVs were also found in cattle from intensive livestock farming without any contact with sheep. Because OaPV DNA was detected in both grass hay and corn silage, it is conceivable that these feed may be the viral sources.

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Short title: OaPV transmission in cattle

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Summary

Ovine papillomavirus (OaPV) comprises four genotypes; OaPV1, OaPV2, and OaPV4 are fibropapillomaviruses within the genus*Delta* -papillomavirus (*Delta-* PV*)* , whereas OaPV3 is an epitheliotropic virus that belongs to the genus*Dyokappa* -papillomavirus (*Dyokappa-* PV*)* . To date, all of them have been known to infect sheep only. OaPV1, OaPV2, and OaPV4 have been associated with ovine cutaneous and mucosal fibropapillomas, while OaPV3 is a key factor in the squamous cell carcinoma (SCC) pathway of the sheep skin. Peripheral blood mononuclear cell (PBMC) samples obtained from 128 cattle at public slaughterhouses were investigated using droplet digital polymerase chain reaction (ddPCR). ddPCR is a new-generation PCR technique that enables accurate and absolute quantification of target molecules with high sensitivity and specificity. All OaPVs were detected by identification and quantification of nucleic acids using specific fluorescent probes. Of 128 PBMC samples, 100 (˜78%) showed OaPV infections. Further, 42, 35, and 23 PBMC samples showed single, double, and triple OaPV infections, respectively. OaPV1 was responsible for 22 single infections, OaPV2 caused 16 single infections, and OaPV3 and OaPV4 caused two single infections each. OaPV1 and OaPV2 were the most frequent ovine viruses in dual and triple infections. In many PBMC samples, both ovine *Delta-* PV and *Dyokappa* -PV were found to be transcriptionally active, as shown by the detection and quantification of E5 oncogene transcripts for OaPV1, L1 transcripts for OaPV2, E6 and E7 transcripts for OaPV3, and E6 for OaPV4. OaPVs were found in the blood samples from cattle that shared grasslands rich in bracken ferns known to contain immunosuppressant substances. Furthermore, OaPVs were also found in cattle from intensive livestock farming without any contact with sheep. Because OaPV DNA was detected in both grass hay and corn silage, it is conceivable that these feed may be the viral sources.

Keywords: Cross-species transmission; Ovine papillomaviruses; cattle; blood; grass hay; corn silage.

Introduction

Papillomaviruses (PVs) are small, non-enveloped, double-stranded DNA viruses infecting mucosal and cutaneous epithelia of mammals, reptiles, birds, and fish (IARC, 2007; Willemsen et al., 2020). As part of the commensal flora, these viruses can be found in the healthy skin and mucosa in a latent state; reactivation occurs following the loss of immunity, resulting in a persistent infection which causes oncogenic risk, with occurrence of tumors at several body sites (Sichero et al., 2019; Strickley et al., 2019).

Ovine papillomavirus (OaPV) infections occur in sheep and are caused by four oncogenic genotypes. OaPV1, OaPV2, and OaPV4 belong to the genus *Delta* -PV, while OaPV3 belongs to the genus *Dyokappa-* PV (http://pave.niaid.nih.gov/). Ovine *Delta-* PV is characterized by marked tropism for both mesenchymal and epithelial cells (Tore et al., 2017), whereas OaPV3 exclusively infects epithelial cells (Alberti et al., 2010). OaPVs have sporadically been associated with ruminal fibropapillomas, papillomas, papillomatosis, and fibropapillomas of the skin (Gibbs et al., 1970; Vanselow et al., 1982; Norval et al., 1985; Trenfield et al., 1990; Tilbrook et al., 1992; Hayward et al., 1993; Uzal et al., 2000). Although it has been suggested that OaPVs may be responsible for the progression of cutaneous papillomas to squamous cell carcinomas (SCCs) in sheep (Vanselow et al., 1982), a novel OaPV, namely OaPV3, was only recently identified in a high number of SCCs in sheep, suggesting that OaPV3 could represent a key infectious agent in the onset of SCC in ovine species (Alberti et al., 2010; Vitiello et al., 2015). OaPV3 and OaPV4 are well-characterized molecularly, as they are the only OaPVs identified in tumor samples from sheep. Indeed, it has been shown that the E6 and E7 oncogenes of OaPV3 and OaPV4 can immortalize primary sheep keratinocytes and regulate the levels of proliferative proteins such as cyclin A and cyclin-dependent kinases (CDKs). However, it has been suggested that only OaPV3 E7 can strongly promote the cleavage and degradation of ovine retinoblastoma protein (pRb) (Tore et al., 2019). Calpain-mediated cleavage of pRb may result in the dysregulation of E2F transcription factors, which play crucial roles in the cell cycle, cell proliferation, and viral replication (Darnell et al., 2007; Scarth et al., 2021). OaPV1 and OaPV2 are not well characterized molecularly so far; however, it has been postulated that they could be associated with tumors in sheep. DNA sequences related to OaPV2 E5 have been found in the mass of the buccal cavity of a pig, suggesting that similar to bovine *Delta* -PVs,

ovine fibropapillomaviruses may also be responsible for cross-species transmission (Munday et al., 2020). Unlike OaPV3 that induces cell transformation by the E6 and E7 oncoproteins (Tore et al., 2019), ovine *Delta* -PVs may exert their main oncogenic activity through the oncoprotein encoded by the E5 gene, as verified in most artiodactyl fibropapillomaviruses (Munger and Howley, 2002). OaPV1, OaPV2, and OaPV4 are fibropapillomaviruses and belong to the *Delta* -PV clade, which is known to encode the most highly conserved E5 oncoproteins (Van Doorslaer, 2013); this is likely because of the integration of the E5 ORF in the *Delta*- PV genus occurring between 65 and 23 million years ago (Garcia-Vallvé et al., 2005).

Recently, the first systematic research on the molecular epidemiology of OaPV infection was conducted in sheep and revealed a divergent geographical prevalence of OaPV genotypes. Furthermore, this survey showed a high prevalence of OaPV infection since OaPV DNA was found in up to 76.4% of the peripheral blood of apparently healthy sheep (De Falco et al., 2021b).

This study aimed to provide evidence of a novel cross-species transmission and infection by OaPVs which were detected, quantified, and found to be expressed in the peripheral blood mononuclear cells (PBMCs) of cattle.

Materials and Methods

Ethics statement

We did not perform any animal experiments in this study. All samples were collected postmortem from slaughterhouses, and hence, ethics approval was not required.

DNA Extraction from blood and matrix samples

Blood samples of 128 healthy cattle aged 2-14 years were collected from regions of southern Italy (Basilicata, Calabria, Campania). All animals were pasture-free, except for 30 cattle from intensive livestock farming. Blood samples harvested in ethylenediaminetetraacetic acid (EDTA)-acid-containing vacutainers were obtained from healthy cattle at public slaughterhouses. Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Wilmington, DE, USA) according to the manufacturer's instructions. Furthermore, DNA was extracted from samples of grass hay and maize silage (used as feed for cattle) obtained from intensive livestock farming. The DNeasy plant Mini Kit (Qiagen, Wilmington, DE, USA) was used for the extraction of hay and maize DNA, according to the manufacturer's instructions.

Positive Controls

The positive controls for *Delta* and *Dyokappa* OaPVs were obtained as previously reported (De Falco et al., 2021b).

ddPCR

Table 1 lists the primers and probes used for ddPCR. Primers and probes were obtained as a mixture containing a primer-to-probe ratio of 3.6 (final concentration of 900 nM of each primer and 250 nM of probe). For ddPCR, a Bio-Rad QX100 ddPCR system was used according to the manufacturer's instructions. The reaction was performed in a final volume of 22 µL and contained 11 µL of ddPCR Supermix for Probes (2X; Bio-Rad Laboratories, Hercules, CA, USA), 1 µL of OaPVs primer and probe mixture, 7 µL of DNA samples (corresponding to 100 ng), and 3 µL of DNAase-free water. The plate containing the reactions was subsequently transferred to an automated droplet generator (AutoDG; Bio-Rad Laboratories, Hercules, CA, USA). AutoDG added 70 µL of droplet generation oil to each well, and each sample was partitioned into approximately 20,000 stable nanodroplets. The droplet generator transferred each row of 8 droplet emulsion (40 µL) champions into a new 96 well PCR plate, which was subsequently coated with a pierceable film heat-sealed using a PX1 PCR Plate Sealer (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplification was performed using a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following thermal profile: hold at 95°C for 10 min, 40 cycles of 94°C for 30 s, 58°C for 1 min, 1 cycle at 98°C for 10 min, and ending at 4°C. After amplification, the plate was loaded onto a droplet reader (Bio-Rad Laboratories, Hercules, CA, USA), and the droplets from each well of the plate were read automatically. The data were

analyzed using the QuantaSoft analysis tool (Bio-Rad Laboratories, Hercules, CA, USA). Poisson statistics were used to calculate the absolute concentration of the OaPV DNA in each sample. A manual threshold line was used to discriminate between positive (blue) and negative (gray) droplets. There were also differences in the fluorescence amplitude range of the background (negative) droplets among the OaPV samples: 1,000–2,500 for OaPV1 E5, 3,500–7,000 for OaPV2 L1, 500–2,700 for OaPV3 E6, 2,000–7,000 for OaPV3 E7, and 1,000–4,000 for OaPV4 E6. Therefore, the ddPCR results could be directly converted into copies/µL in the initial samples simply by multiplying them by the total volume of the reaction mixture (22 µL) and then dividing that number by the volume of the DNA sample added to the reaction mixture (7 µL) at the beginning of the assay. Each sample was analyzed in duplicate. According to previous studies on PV detection and quantification using ddPCR (De Falco et al., 2021a; 2021b; Jeannot et al., 2016; Jeannot et al., 2021), blood samples were considered OaPV-positive in the presence of at least three positive droplets at the same amplitude as positive controls. A sample was considered OaPV-negative when fewer than three droplets or no droplets containing OaPV amplicons were observed.

Limit of Detection (LoD) Determination

The four OaPV genes were detected using ddPCR standard curves of the positive controls used in the serial dilutions. A calibration curve of the positive sample dilutions (log10) was plotted against the number of PCR cycles. The linear range was determined by diluting the positive controls from 10^5 to 10^{-1} copies/µL, detecting each dilution thrice, taking the average value, and correlating the result with the theoretical value. The lower detection limit obtained by ddPCR, with values <1 copies/µL, indicated high sensitivity.

qPCR

The primers and probes used for the real-time qPCR assay are listed in Table 1. The qPCR reaction mixture (total volume of 20 µL) was prepared by adding 7 µL of template (100 ng genomic DNA), 10 µL of 2X SsoAdvanced^{TM} Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 1 µL of target probe (FAM)/primer mix. The DNA quality and concentration were evaluated using a NanoDrop spectrophotometer (Thermo Scientific, MA, USA). Four separate PCR were performed using the CFX96 Real-Time System of the C1000 Touch^{TM} Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycling conditions were as follows: 95°C for 5 min, and 40 cycles of 95°C for 15 s and 58°C for 30 s. Each sample was analyzed in duplicate, and positive as well as negative controls were included in all runs. Data acquisition and analysis were performed using CFX Maestro^{TM} (BioRad Laboratories, Hercules, CA, USA) software.

PCR

PCR was performed with DNA isolated from the blood, grass hay, and maize silage samples using EconoTaq PLUS (Lucigen, WI, USA) and the OaPV primers (see Table 1) according to the manufacturer's instructions. Conditions for PCR were as follows: 94°C for 2 min; 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 20s; final extension at 72°C for 5 min. All the amplicons were sequenced.

Statistical Analysis

McNemar's test for two related binomial proportions (conditional) was used to evaluate the agreement between two tests performed on the same animals. To evaluate the actual differences in the prevalence of the four types of PVs in the same animals, the Cochran-Armitage test was performed. P <0.05 was considered to be statistically significant.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from 34 blood samples using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA was removed from the RNA samples using RNase-free DNase I (Fermentas Life Sciences, Thermo Fisher Scientific, MA, USA). One microgram of the Total RNA was used to generate a single strand of cDNA using the QuantiTect Reverse Transcription Kit (Qiagen TM, Germany), according to the manufacturer's instructions. PCR was performed on samples with

and without reverse transcriptase (RT) added to the reaction mix, using the primers reported in Table 1. The conditions used for PCR were: 94 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. All the amplicons were sequenced.

One-Step reverse transcription (RT)-ddPCR

Total RNA was extracted from 34 healthy cows (as negative controls) as previously reported. 100 ng of total RNA was used for One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The reaction was performed in a final volume of 22 µL, containing 11 µL of ddPCR Supermix 2x for Probes, 1 µL of primer and probe mix for OaPVs (Table 1), 2 µL reverse transcriptase, and 1 µL DTT. The plate was transferred to an automated droplet generator (AutoDG, Bio-Rad Laboratories, Hercules, CA, USA) as described above. PCR amplification was carried out on a T100 Thermal Cycler (Bio-Rad Laboratories Hercules, CA, USA) with the following thermal profile: 50°C for 60min, 95°C for 10 min, 40 cycles of 94°C for 30 s, 58°C for 1 min, 1 cycle at 98°C for 10 min, and ending at 4°C. After amplification, the plate was loaded onto a droplet reader (Bio-Rad Laboratories, Hercules, CA, USA), and the droplets from each well of the plate were read automatically. Therefore, the ddPCR results could be directly converted into copies/µL in the initial samples simply by multiplying them by the total volume of the reaction mixture (22 µL) and then dividing the number by the volume of the RNA sample added to the reaction mixture (5 µL) at the beginning of the assay. Each sample was analyzed in duplicate.

Sequence Analysis

PCR products from DNA and cDNA were purified using a Qiaquick PCR purification Kit (Qiagen TM, ME, DE) and bidirectionally sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) following the manufacturer's recommendations. Sequences were removed with a DyeEx_2.0 spin kit (Qiagen TM, DE) and run on a SeqStudio Genetic Analyzer (Thermo Fischer Scientific, CA, USA). Electropherograms were analyzed using Sequencing analysis v5.2 and sequence scanner v1.0 software (Thermo Fischer Scientific, CA, USA). The obtained sequences were compared to other sequences in GenBank using BLAST.

Results

OaPV DNA was detected in 100 of 128 PBMC samples (˜78%) examined through ddPCR. Detection and quantification of OaPVs were performed by ddPCR. In particular, OaPV1 DNA quantification ranged from 0.22 to 34.4 copy number/µL; OaPV2 DNA from 0.23 to 14.92 copy number/µL; OaPV3 DNA showed a range from 0.25 to 6.22 copies per µL, and finally OaPV4 DNA from 0.25 to 12.03 copies per µL. These detailed results are listed in Figure 1 SuppInfo.

Among 100 OaPV positive samples, single infections were detected in 42 (42%) using ddPCR, whereas only 18 (18%) were detected using qPCR. Differences between the two molecular methods in detecting OaPV DNA were statistically significant, as shown by McNemar's test ($p < 0.05$).

OaPV1 and OaPV2 infections were the most representative being found in 22 (52.4%) and 16 (38%) single infections by ddPCR. On the other hand, qPCR revealed OaPV1 and OaPV2 infections in 9 (50%) and 8 (44.4%) samples, respectively. Hence, OaPV1 and OaPV2 infections were the most prevalent single infections as per results obtained from both the methods. OaPV3 and OaPV4 DNAs were found in two single infections (4.8%) by ddPCR, while qPCR revealed only a single OaPV3 infection (5.6%). No OaPV4 infection was detected by qPCR. Figure 1 summarizes the results obtained by ddPCR.

Differences in OaPV DNA genotype detection were statistically significant, as shown by the Cochran-Armitage test ($p < 0.05$).

Table 2 summarizes the results of the coinfection studies. Coinfections were observed in 58 out of 100 (58%) positive samples by ddPCR. In particular, 35 (35%) were dual infections, and 23 (23%) were triple infections. Dual coinfections by OaPV1/2 were the most frequent being observed in 23/35 (˜66%); coinfections by

OaPV1/3, OaPV2/3, OaPV2/4, and OaPV3/4 were more rarely detected. Coinfections with OaPV1/2/3 were the most frequent triple infections, as observed in 18/23 samples (˜78%). OaPV1/2/4 infections have also been reported. qPCR failed to detect most of the multiple coinfections and identified only seven dual infections. Furthermore, qPCR revealed only one genotype in many dual and triple coinfections confirmed by ddPCR. Triple coinfections were not observed by qPCR. However, ddPCR revealed that OaPV1 was the most prevalent genotype in multiple coinfections being detected in 52 of them. OaPV2, OaPV3, and OaPV4 were detected in 51, 29, and 7 samples, respectively. In the same multiple coinfections, qPCR revealed presence of the OaPV1, OaPV2, OaPV3, and OaPV4 genotypes in 13, 7, 2, and 1 samples, respectively.

PCR analysis, using DNA isolated from PBMC samples, detected amplicons using primers specific to all OaPV genotypes. Sequencing revealed the presence of DNA fragments with 100% identity with OaPV1 E5, OaPV2 L1, OaPV3 E7, and OaPV4 E6 DNAs reported in GenBank (accession number: U83594.1., U83595.1., NC_038516.1, and KX954121.1, respectively) (Figure 2).

One-step reverse transcription (RT)-ddPCR was performed on 34 randomized positive samples. We detected and quantified the transcripts of OaPV1 E5 and OaPV2 L1 as well as transcripts of OaPV3 E6, E7, and OaPV4 E6, which showed that all OaPV genotypes can be transcriptionally active in healthy cattle. The Bio-Rad system quantified mRNA in copies per µL. Samples were considered positive if they had at least three or more positive droplets at the same amplitude as the positive control (Figure 3). Details of this investigation are reported in Figure 2 SuppInfo.

Furthermore, we performed RT-PCR analysis of RNA from the PBMC samples. We detected amplicons, the sequencing of which showed 100% identity with OaPV1 E5, OaPV2 L1, OaPV3 E6 and E7, and OaPV E6 mRNAs reported in GenBank, thus validating the one step RT-ddPCR results (Figure 4).

OaPV coinfections were most prevalent in cattle that shared grasslands with sheep. In cattle from intensive dairy farms without any apparent contact with sheep, double coinfections with OaPV1 and OaPV2 were also observed (detected in 11 out of 30 examined PBMC samples). OaPV1 E5 and OaPV2 L1 DNAs were found in feed composed of grass hay and corn silage, which are known to be fed. ddPCR detected and quantified high copy numbers of OaPV1 DNA as it was found in grass hay samples (from 4.2 to 7.7 copies per µL) as well as in feed composed of maize silage (from 3.43 to 5.7/µL copy number). OaPV2 DNA was also detected in grass hay (up to 14.4 copies per µL) and corn silage samples (up to 10.9 copies per µL) (Figure 3A SuppInfo). PCR analysis performed on all these matrix samples revealed amplicons, and the sequencing of obtained DNA fragments showed 100% identity with OaPV1, and OaPV2 DNA deposited in GenBank (Figure 3B SuppInfo).

Discussion

Oncogenic OaPVs infect only ovine species. Accordingly, all benign and malignant OaPV-related tumors have been described exclusively in sheep. OaPVs have never been associated with any pathology in other domestic animals. This study shows, for the first time, that cross-species transmission by both *Delta* and epitheliotropic *Dyokappa*OaPVs occurs between sheep (*Ovis aries*) and cattle (*Bos taurus*). All OaPV genotypes were detected through both DNA detection and transcriptionally active forms in PBMC samples from healthy cattle. Transcripts of E oncogenes as well as the L1 gene were peculiar molecular findings of this study, which suggested that the biological properties of OaPVs might be characterized by inducing both abortive and productive infections in cattle.

The scant information on the epidemiology of OaPV infections is the reason for the poor understanding of the biological significance of these viruses in cattle. PV transmission by blood in sheep and other species appears to be a key event in their pathology and epidemiology (Cutarelli et al., 2021b). It has been suggested that the blood infected with PV yields infections at permissive sites with detectable viral DNA, RNA transcripts, and viral proteins (Cladel et al., 2019; Syrjänen and Syrjänen, 2021). Similar to humans, it is conceivable that ecological factors can influence the virulence of several PVs from different genera, and the concomitant ecological changes in different hosts linked to the human domestication of farm animals, including sheep, may have increased their susceptibility to OaPV cross-species transmission and/or simply increased the frequency

of physical contact to grant OaPVs improved access to a potential new host.

PVs have a long history of co-divergence with their hosts, and hence, these viruses are relatively more host-specific than other viruses (Geoghegan et al., 2017). Indeed, to date, Bovine *Delta* -PVs have been the only PVs responsible for documented cases of natural cross-species transmission leading to carcinogenetic events via oncoproteins encoded by E genes (IARC, 2017; Roperto et al., 2013). Most OaPV-harboring cattle in this study have in common highlands rich in bracken ferns with sheep that live in the same geographical zones. Close physical proximity and/or sharing of grazing lands may be a prerequisite for PV types to cross host-species barriers, as suggested by the detection of various BPVs in other hoofed domestic animals (de Villiers et al., 2004; Cutarelli et al., 2021; Roperto et al., 2021). It is conceivable that animal husbandry practices and/or mammalian sympatry may contribute toward the cross-species transmission of OaPVs. This is corroborated by the fact that a high prevalence of OaPVs was found in cattle sharing large enclosures with sheep, which facilitates direct and indirect contacts. Therefore, our study strengthens the assumption that cross-species transmission may occur among related hosts inhabiting the same geographic areas (Parrish et al., 2008). Successful cross-species transmission has been suggested to occur among phylogenetically related hosts, likely because they share fewer divergent cell receptors (Murthy et al., 2013). Indeed, it is believed that the closer the phylogenetic relationship between hosts, the more likely it is that a pathogen will be able to jump between them with appropriate exposure. Immunosuppressants of bracken coupled with more frequent exposure between sympatric hosts may help OaPVs jump host species, resulting in host switching.

OaPVs have also been detected in cattle from intensive dairy farms without any apparent contact with sheep. The cows were fed grass hay and corn silage prepared with grass and maize grown using irrigation water from the Volturno River. PVs have recently been detected and quantified in surface water of rivers (Iaconelli et al., 2015). It has been shown that PVs can be detected in vegetables and irrigation water and long-term consumption of HPV-polluted water can be associated with cell transformation (Ghaffar et al., 2018; Itarte et al., 2021). Several PV genotypes of different genera are known to be responsible for oral infection; however, the information on associated risk factors is still limited (Wong et al., 2018). It is possible that polluted irrigation water may have played an overlooked role in the OaPV epidemiology. The molecular findings of this study appear to strengthen our suggestions since OaPV DNA has been detected and sequenced in hay as well as corn silage samples. However, further studies on virus isolation from these feed should be conducted to better understand the actual risk of virus transmission from feed. It is well known that PVs can survive without significant loss of infectivity during desiccation, as well as at low pH and high temperatures, chemical features that characterize these feed (Roden et al., 1997; Nielsen et al., 2021). Furthermore, a recent scientific report by the European Food Safety Authority (EFSA) showed that some viruses, including African swine fever virus (ASFV), can be transmitted through feed based on hay and maize (Nielsen et al., 2021).

As the number of cross-species transmissions continues to rise and viral diseases pose a continual threat to animal populations, understanding the ecological diversity of OaPV prevalence and genotype distribution among new host species in different geographical regions remain essential. The need to understand how PV transmit within a given species, as well as to new host species, has become increasingly important as the cross-species transmission of viruses from one host species to another is responsible for the majority of emerging infections that can profoundly affect animal health (Geoghegan et al., 2017).

Finally, both circulating OaPV DNA and OaPV RNA have been reported in the blood samples of cattle found healthy by ante- and post-mortem anatomo-clinical observations, confirming that blood represents an important primary route of PV infection and that OaPVs can disseminate to any organ via the bloodstream. We detected and sequenced OaPVs in the urinary bladder of healthy cattle (personal observations), which supports the hypothesis that OaPVs may contribute to the composition of the normal bladder microbiota of cattle. BPV and HPV DNA have also been reported in the blood of healthy cattle (De Falco et al., 2021a) and asymptomatic blood donors (Vergara et al., 2019). It has been suggested that there is an actual likelihood that HPVs could reach epithelial target sites in the blood, which might explain how and why HPVs are associated with tumors of several organs (Cladel et al., 2019; Syrjänen and Syrjänen, 2021 Vergara et al., 2019; Conceição Gomes Nascimento et al., 2021). Accordingly, PV transmission by blood represents

a conceptually novel idea, and if accepted more generally, this would change the current thinking about the modes of PV spread, including HPV within the host (Cladel et al., 2019; Roperto et al., 2013; Syrjänen and Syrjänen, 2021). In this context, a naturally occurring OaPV infection may be an additional animal model that sheds light on the PV biology, including issues related to viral tropism in the One Health approach.

Data Availability All relevant data are within the manuscript and its *Supporting information* files.

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Figure legends

Figure 1. Detection rates of OaPV single infections. Differences were significant after Cochran-Armitage test ($p < 0.05$).

Figure 2. (A) Electrophoresis of PCR products for evaluating OaPV1 E5, OaPV2 L1, OaPV3 E7, and OaPV4 E6 DNA in bovine PBMC samples. MW: DNA molecular weight marker (100 bp). C: PCR negative control. (B), (C), (D), and (E):100% identity between the sequences of the amplicons OaPV1 Seq, OaPV2 Seq, OaPV3 Seq, and OaPV4 Seq, and the sequences reported in GenBank, respectively (accession number: U83594.1; U83595.1; NC_038516.1; and KX954121.1).

Figure 3. One Step ddPCR. The rain plots for the OaPV1 E5, OaPV2 L1, OaPV3 E6 and E7, and OaPV4 E6 are shown. Representative samples have positive droplets at the same amplitude as the positive control for each OaPVs.

Figure 4. (A) Electrophoresis of RT-PCR products to evaluate OaPV1 E5, OaPV2 L1, OaPV3 E6 and E7, and OaPV4 E6 mRNA expression in PBMC samples. MW: DNA molecular weight marker (100bp); C+: PCR positive control. PCR was performed on PBMC samples with (RT+) and without (RT-) the addition of reverse transcriptase to the reaction mixture, using the same amount of RNA. (B) The amplicon sequences showed 100% identity with the respective sequences reported in S1 Fig. Furthermore, 100% identity between the OaPV3 E6 cDNA amplicon and the OaPV3 E6 sequence reported in GenBank (accession number: NC_038516.1) is shown.

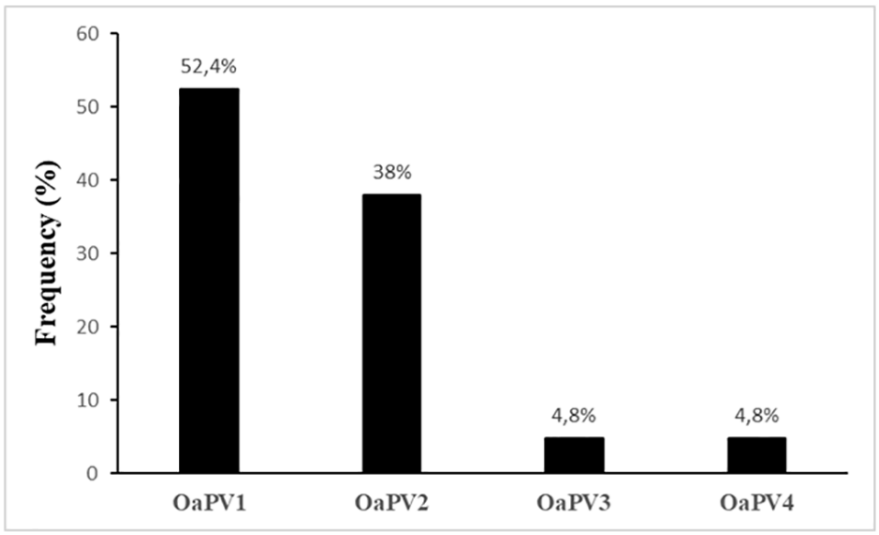
Figure 1 SuppInfo. Detection and quantification of OaPV DNA using ddPCR. The Bio Rad system quantified DNA copies per µL. Bov = bovine; N = negative

Figure 2 SuppInfo. Detection and quantification of mRNA using one-step reverse transcription (RT)-ddPCR. The Bio Rad system quantified mRNA copies per µL. Bov = bovine; N = negative

Figure 3 SuppInfo. (A) . Representative positive samples and a positive control for the OaPV1 E5 and OaPV2 L1 assessed by ddPCR in grass hay and maize silage samples. Positive samples have droplets at the same amplitude as the positive control. Amplitude of OaPV1 E5 ranged from 1.500 to 5.500; amplitude of OaPV2 L1 ranged from 1.500 to 11.500. (B). PCR products of the above samples. MW: DNA molecular weight marker (100bp). C+: PCR positive control; C-: PCR negative control. The amplicon sequences showed 100% identity with the respective sequences reported in GenBank (see Figure 2).

Table 1 shows the primers and probes used for the detection and quantification of OaPVs.

Table 2- Genotype coinfections evaluated by ddPCR with number and related percentages of their combination are shown.



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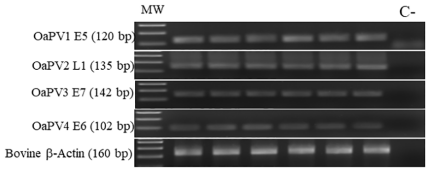
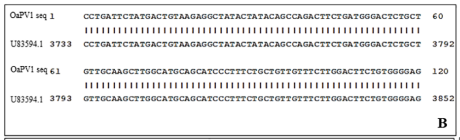
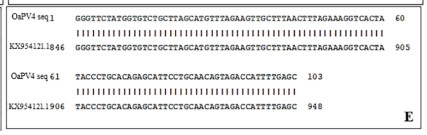
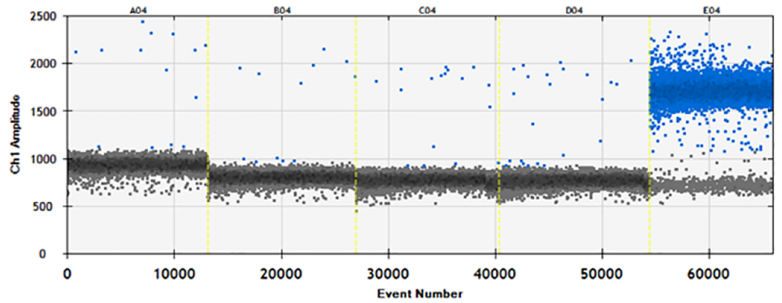
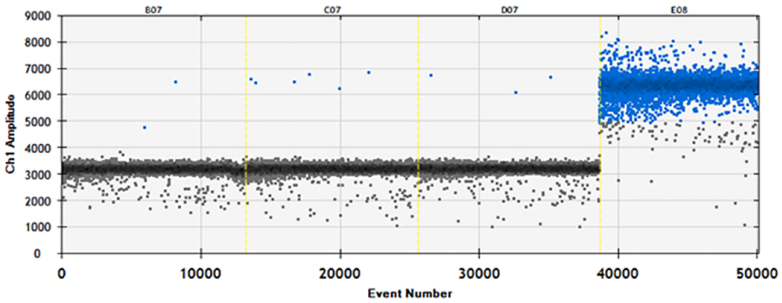




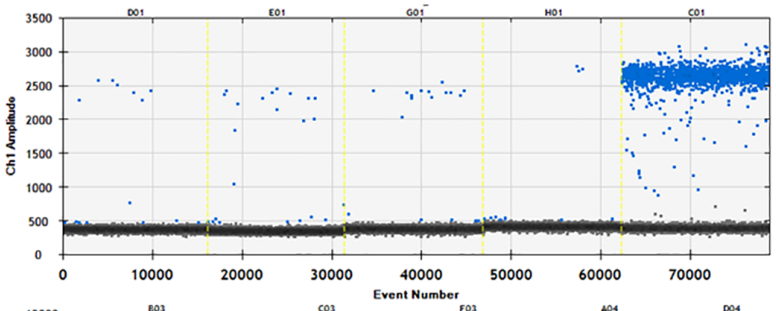




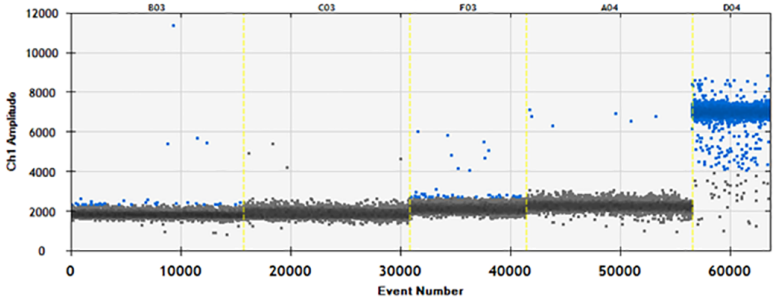
OaPV1



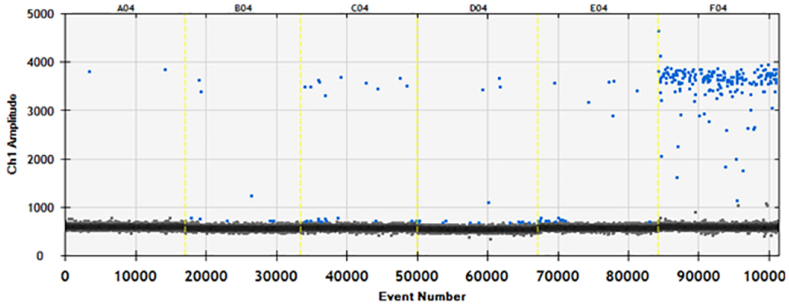
OaPV2



OaPV3
E6



OaPV3
E7



OaPV4

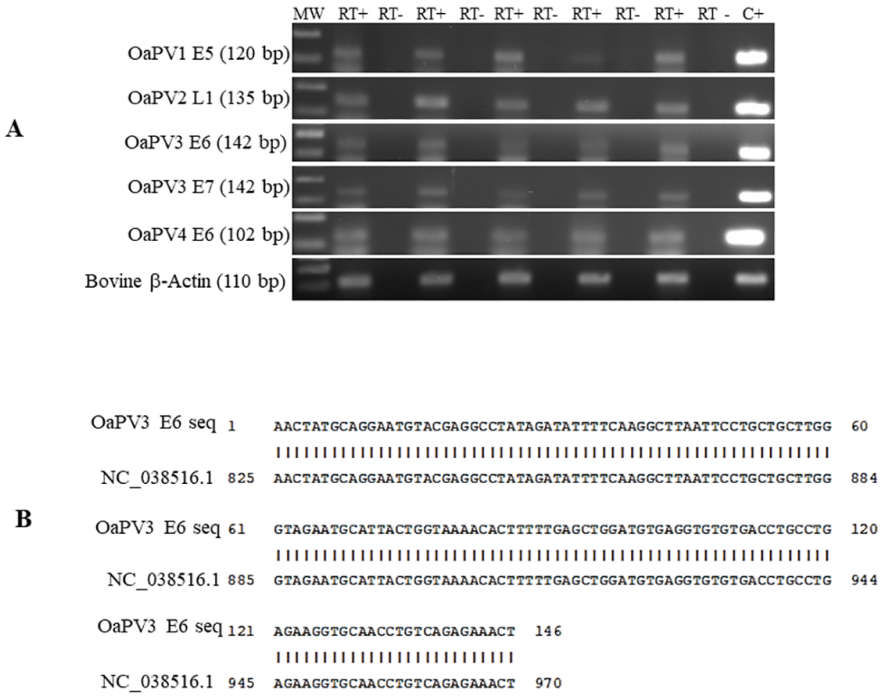


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Table 2.docx available at https://authorea.com/users/319003/articles/581510-evidence-of-a-novel-cross-species-transmission-by-ovine-papillomaviruses