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Digital droplet PCR-based detection and quantification of ovine papillomavirus DNA from the vaginal virobiota of healthy mares

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There are four genotypes of ovine papillomaviruses (OaPVs): OaPV1, OaPV2, and OaPV4, which are ovine delta papillomaviruses responsible for epithelial and mesenchymal cell infections, and OaPV3, an epitheliotropic Dyokappapapillomavirus associated with cutaneous tumors in sheep, including squamous cell carcinoma. Vaginal swabs of healthy mares were evaluated for the presence of PVs to investigate whether the vaginal virobiota of asymptomatic mares harbored OaPVs. High-performance digital droplet polymerase chain reaction (ddPCR) was used to quantitatively detect OaPV types 1–4 DNA in 94 vaginal swabs collected at the National Reference Center for Veterinary and Comparative Oncology (CEROVEC), Genoa, Italy. All samples were comparatively evaluated for OaPV DNA loading using real-time quantitative PCR. ddPCR detected OaPV DNA in 25 vaginal swab samples (26.6%), whereas qPCR revealed 13 vaginal swabs (11.7%). Differences between the two molecular protocols were determined to be statistically significant using McNemar's test ($p < 0.0005$). The detected OaPV types were OaPV1 and OaPV3. Both methods failed to detect OaPV2 or OaPV4 DNA, which could be attributed to the limited number of samples examined. OaPV1 is the most prevalent OaPV in equine vaginal virobiota . This study is the first to provide evidence of the presence of OaPV DNA in vaginal swabs of healthy mares. This comparative detection approach underscores the superior sensitivity of ddPCR over qPCR.

Keywords Droplet digital polymerase chain reaction, Healthy mares, Ovine papillomavirus, Real time quantitative polymerase chain reaction, Vaginal virobiota, Vaginal swabs

Papillomaviruses (PVs) are small, nonenveloped, double-stranded DNA viruses that infect the skin and mucosa of various animals, including fish, reptiles, birds, and mammals, in a species-specific manner[1]. The viral genome varies from 5,748 to 8,607 bp. The genome comprises three functional regions. The early region encodes proteins involved in transcription, replication, and transformation; the late region encodes capsid proteins L1 and L2[2].

Ovine PVs, classified as *Ovis aries* PVs (OaPVs), comprise four genotypes assigned to two genera. OaPV1, OaPV2, and OaPV4 are fibropapillomaviruses that belong to the genus *Deltapapillomavirus*, whereas OaPV3 belongs to the genus *Dyokappapapillomavirus*[3]. Despite their long coevolutionary history with their hosts, some PVs are pathogenic to their natural host species[2]. OaPVs are associated with benign and malignant tumors in sheep[4,5]. OaPV1, OaPV2, and OaPV4 are known to infect both epithelial and mesenchymal cells[6], whereas OaPV3 displays transforming activity in keratinocytes, suggesting that OaPV3 could be a key infectious agent in the onset of squamous cell carcinoma in ovine species[7]. However, it has been shown that OaPVs can be responsible for cross-species transmission and infection. OaPV DNA and its transcripts have been found in cattle, both in the blood of healthy animals[8] and in bladder tumors[9,10]. Furthermore, transcriptionally active OaPVs have been detected both in the commercial semen of healthy stallions[11] and in semen samples associated with equine sarcoid[12]. It has been suggested that ovine delta-PVs exert their main oncogenic activity through

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Genotypes	Forward 5′ 3′	Reverse 5′ 3′	Probe	Region	Size-bp
OaPV1	CCTGATTCTATGACTGTAAGAGGC	CTCCCCACAGAAGTCCAAG	TGCAACAGCAGAGTCCCATCAGAAG FAM	ORF E5	119[8]
OaPV2	AGTTCCCGCTCTGATTTACC	ATGGCGGACGTATACTTGTTC	ATTGCCAGCAGTCTCCTCAGTCATTC FAM	L1	134[8]
OaPV3	AGCCCACACTCCCTGATATAG	TTCAGTCTTTGACAGCACCTC	AGCAACCAGCACTGTACACGCTAT FAM	E7	145[8]
OaPV4	GGGTTCTATGGTGTCTGCTTAG	GCTCAAAATGGTCTACTGTTGC	CAGGAATGCTCTGTGCAGGGTATAGTG FAM	E5	102[8]

Table 1. Primers and probes used to detect OaPVs in ddPCR and qPCR.

	OaPV1	OaPV3
ddPCR	92% (23/25)	8% (2/25)
qPCR	44% (11/25)	8% (2/25)

Table 2. Percentages of OaPV1 and OaPV3 DNA detected by digital droplet polymerase chain reaction (ddPCR) and real-time quantitative PCR (qPCR). Statistical differences were significant as shown by Fisher's exact test ($p < 0.0001$). Neither protocol detected OaPV2 or OaPV4 DNA.

an oncoprotein encoded by the *E5* gene, as verified in most artiodactyl fibropapillomaviruses[13]. The E5 protein transforms cells by activating the cellular platelet-derived growth factor β receptor (PDGFβR), although there may be additional minor, alternative transforming pathways[14,15]. All fibropapillomavirus E5 proteins are highly conserved oncoproteins, likely because of the integration of the E5 ORF into the genome of an ancestor of the genus *Deltapapillomavirus* occurring between 65 and 23 million years ago[16]. E5 oncoproteins exhibit strict conservation of glutamine at position 17, aspartic acid at position 33, and cysteines at positions 37 and 39, all of which are required for the biological activity of E5 proteins[17]. Indeed, complex formation and biological activity are crucially dependent on glutamine at position 17 and aspartic acid at position 33 of E5 and Lys-499 and Thr-513 in the PDGFβR throughout fibropapillomavirus evolution[18]. The E5 oncoprotein specifically binds to the transmembrane domain of the PDGFβR and induces receptor dimerization and activation, resulting in receptor autophosphorylation and the initiation of a mitogenic signaling cascade[18]. OaPV3 induces cell transformation via E6 and E7 oncoproteins. In particular, OaPV3 E7 strongly promotes the cleavage and degradation of the ovine retinoblastoma protein (pRb)[19]. 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[20,21]. Experimental data have shown that calpain family members work cooperatively to regulate their protease activities[22]. A proteomic study revealed the overexpression of proteolytically active calpain3 in papillomavirus-associated bladder tumors[23]. Activated calpain3 may trigger a calpain cascade, thereby facilitating calpain1 activity, resulting in overexpression of the E_2F_3 transcription factor recently shown in spontaneous OaPV infection, leading to bladder tumors in cattle[10].

The droplet digital polymerase chain reaction (ddPCR) is an improved method of conventional PCR that can amplify and directly quantify DNA or RNA[24]. As it is more sensitive than classical approaches, including real-time quantitative PCR (qPCR)[25,26], it can detect small amounts of nucleic acids that may otherwise be undetectable. Similar to pathogens that cause latent infections, PV concentrations in biological matrices are sometimes too low to be determined using traditional methods. Therefore, ddPCR is currently reported as an ultrasensitive and highly precise method for the detection and quantification of papillomavirus nucleic acids in humans[27,28] and domestic animals[11,29–32]. ddPCR allows the precise quantification of very low viral copy numbers, which may permit more reliable monitoring of latent papillomavirus reservoirs (Table 1).

The vaginal microbiome is a dynamic microenvironment that plays an important role in general and reproductive health[33]. Little information is available on the vaginal microbiome of healthy mares[34]. In particular, understanding some unprecedented viruses that compose the vaginal virobiota of mares is important for gaining deeper insights into their molecular epidemiology, territorial distribution, and role, if any, in potential reproductive disorders.

The current study aimed to investigate the ddPCR-based detection and quantification of OaPV DNA in vaginal virobiota from healthy mares.

Results

The frequency of OaPV DNA in vaginal swabs was approximately 26.6% using ddPCR (25/94) and approximately 11.7% using qPCR (11/94). Differences between the two molecular protocols were determined to be statistically significant using McNemar's test ($p < 0.0005$). Of the OaPV-infected vaginal swabs, 92% (23/25) were positive for OaPV1 through ddPCR, whereas qPCR revealed this infection in 44% (11/25) of the samples, and 8% (2/25) were positive for OaPV3 when examined using either ddPCR or qPCR (Table 2). Fisher's exact test showed that the number of positives detected for the *OaPV1* gene using ddPCR (23 positives) and qPCR (11 positives) was significantly different ($p < 0.0001$). Overall, OaPV1 DNA showed a number of copies/µL ranging from 0.1 to 0.5, whereas qPCR detected OaPV1 DNA with a cycle threshold (CT) value varying from 33.3 to 35.0, thus correlating with ddPCR data (Fig. 1). This figure compares the qPCR cycle of quantification and the relative rain

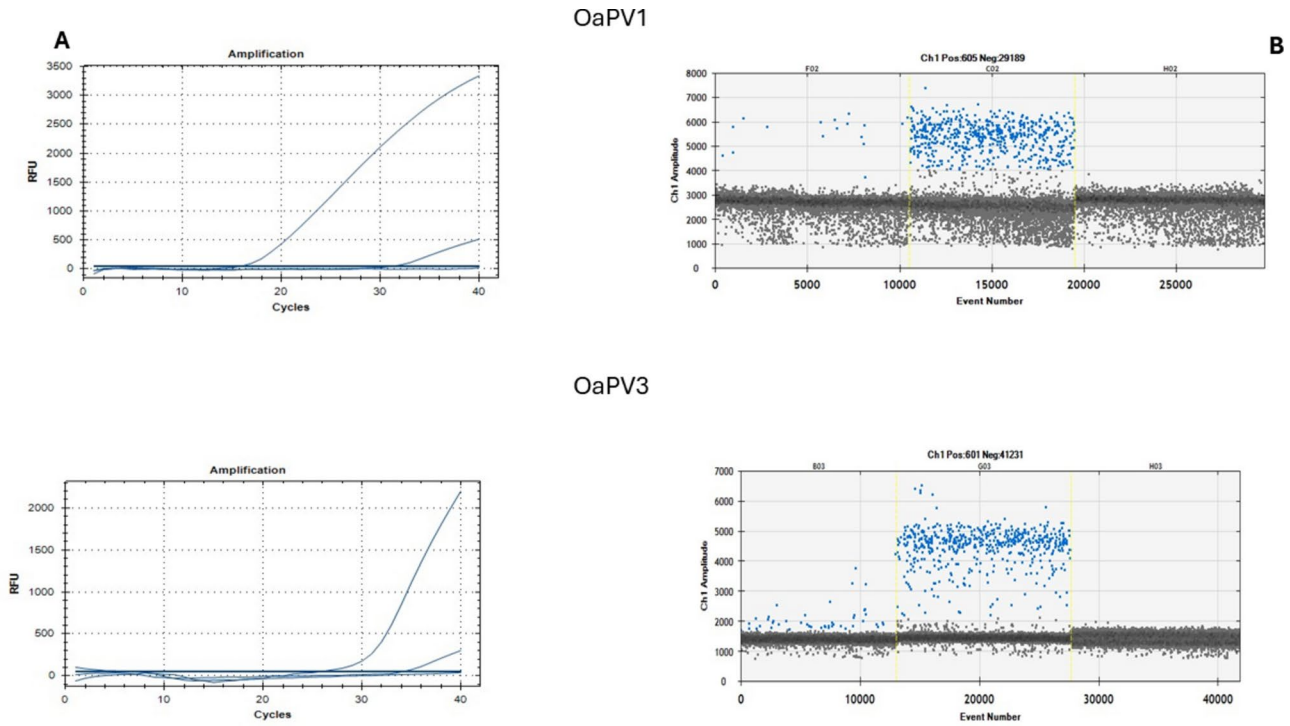

Fig. 1. qPCR curves (**A**) and the relative rain plots of the ddPCR (B) for the OaPV1- and OaPV3 DNA. QuantaSoft screenshots show the ddPCR results. Positive plots are represented in blue, whereas negative droplets are in grey. OaPV1-F02: positive samples; H02: negative control; and C02 is the positive control. OaPV3-B03: positive samples; H03: negative control; and G03 is the positive control.

plots of ddPCR. By contrast, the *OaPV3* gene showed two positive results when both molecular techniques were used. OaPV3 DNA had a copy number/µL of 0.7 and 1.0, respectively, while the CT values were 30.8 and 32.6, respectively. The raw data obtained by ddPCR and qPCR are summarized in Supplementary Table S1.

Discussion

The lower female reproductive tract hosts a complex microbial environment, including viruses whose roles in health and disease are not well understood[35]. Vaginal microbiota refers to the microorganisms inhabiting the vagina. Few studies have specifically addressed the microbiome of the equine vagina. Most of these studies have focused on bacteria and fungi, whereas little is known about the viral community[34–37]. PV are known to be part of the vaginal biota in several animal species[38,39]. Our study showed the presence of OaPV DNA in vaginal swabs of healthy mares, which has never been reported before. OaPV1 was the most prevalent genotype among the OaPVs. OaPV DNA quantification revealed a low copy number/µL compared with OaPV DNA copies detected in the semen of healthy stallions[9] and in association with equine sarcoid[10]. ddPCR has proven to be an accurate and sensitive diagnostic tool as it significantly increases the detection of OaPVs with low viral abundance. Therefore, ddPCR may serve as a reliable diagnostic method for identifying otherwise undetectable infectious agents that compromise the reproductive efficiency of mares. Enhancing diagnostic accuracy is crucial, as mare reproductive performance is relatively low. In addition to maternal age, few risk factors, including infectious factors, have been conclusively identified[40,41].

To date, bovine papillomavirus type 1 and 2 and Equus caballus papillomavirus type 2 have been shown to be part of the viral community of the genital mucosa of healthy mares[42–46]. However, our understanding of PVs infecting horses is limited, as little is known about the mechanisms of oncogenicity, routes of transmission, the existence of a carrier state, or the impact of PVs on host health[42]. Therefore, the biological significance of OaPVs inhabiting the lower female reproductive tract of mares remains to be understood, which limits the ability to draw causal inferences. Transcriptionally active OaPVs have been previously detected and quantified in equine peripheral blood[11], which allows us to suggest that the presence of OaPVs in the lower female reproductive tract of mares may originate via blood spread. However, it is worth remembering that OaPV DNA and RNA have been detected and quantified in the semen of healthy stallions, with OaPV1 being the most prevalent OaPV[11]. Therefore, it is conceivable that semen harboring OaPVs plays a crucial role in OaPV epidemiology and genotype distribution via natural mating and/or insemination. Our findings are corroborated by the fact that infectious pathogens found in the genital tracts of stallions are usually isolated from the genital tracts of mares after natural mating with stallions[47,48]. Therefore, iatrogenic transmission via fomites and/or gynecological procedures must be comprehensively evaluated. Furthermore, although the results are controversial, emerging evidence suggests an association between PVs and female subfertility, as PV infection of the lower genital tract is believed to increase the risk of pregnancy failure and abortion[49]. OaPVs have been shown to infect the uterus and placenta

after transcervical migration from the lower genital tract mucosa and are thus responsible for vertical infection and pregnancy loss in cows[50]. The findings of this study raise an important question that warrants further research into reproduction. The presence of OaPVs in the vaginal swabs of healthy mares may be a potential concern for equine reproductive efficiency, as OaPVs could be an additional risk factor for reproductive disorders in mares. OaPVs can be expressed in equine species, thus causing a novel cross-species transmission[11,12]. Furthermore, OaPV infection was found to be associated with equine sarcoids, with a high rate detected in approximately 35% of the examined samples, which suggests that OaPVs may actually be involved in the pathogenetic mechanisms leading to equine sarcoids[12]. Future studies on genital OaPV infection in mares should be a priority to improve our understanding of the complex relations between horses and their associated virobiota and gain insights into the correlation between OaPV load and pathogenicity. Identification of causal associations between genital OaPV infection in mares and equine reproductive efficiency would require dose–response analyses, as viral load is one of the key indicators of pathogenicity and may be an important factor that positively correlates with OaPV pathogenicity and the ability for persistent infection, similar to other mammalian PV genotypes[51,52].

The current study provides unequivocal evidence and further confirms that OaPVs are responsible for host jumps not only in the Bovidae family[8–10] but also between the closely related orders Perissodactyla and Artiodactyla, which together comprise a diverse clade of ungulates, such as sheep (*Ovis aries*) and horses (*Equus caballus*). It is conceivable that OaPVs have been responsible for cross-species transmission for long time, which argues that, like BPVs[53], OaPV spread between ovines and equines could be a repeated and ongoing phenomenon. The growing number of PV cross-infections suggests that certain PVs may exhibit a broader host range and/or that host switches between different species may occur more frequently than initially suspected. It is conceivable that virus–host coevolution is not a major determinant of OaPV evolution, as is the case in other mammalian PVs. This potential to infect different hosts could explain the highly polyphyletic patterns displayed by many PVs[54].

Conclusion

Our study showed that OaPVs can be found in the vaginal biota of healthy mares. Although eukaryotic viruses have both detrimental and beneficial effects on host health, as they can be both pathogenic and commensal[55], evidence suggests that vaginal PVs could have a negative impact on reproductive health in both domestic animals and humans. It is worth noting that an association has been found between HPV and infertility, as HPV is the most abundant viral agent detected among infertile women[56]. Furthermore, both bovine papillomaviruses belonging to *Deltapapillomavirus* genus and OaPVs have been shown to be associated with pregnancy failure in ruminants[50,57]. Although infectious agents are believed to represent the primary causes of equine reproductive disorders, it is notable that a definitive cause of equine pregnancy failure is determined in fewer than 30% of cases[58]. Control of infectious agents and diseases is believed to be a crucial area of interest, both in current trends and future perspectives[59,60], contributing to improved understanding of reproductive disorders in domestic animals. Therefore, new diagnostic approaches are required to enhance diagnostic accuracy.

Methods

Ethics statement

This study was approved by the Institutional Ethics Committee of Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta (approval number 14047 of 11/28/2019). This study was carried out in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Vaginal swabs were performed in horses admitted to the Veterinary Teaching Hospitals of Turin (OVU) for causes not related to pathologies of the genital system. Permission to collect vaginal swabs was obtained from the animals' owners who were previously informed and in agreement with the purpose and methods used.

Animal sampling

Sterile cytobrushes (Deltalab SLU, Barcelona, Spain) were used to collect vaginal samples from ninety 3- to 21-year-old mares, as previously described[61]. The mares were from several Italian regions, including Piedmont, Umbria, Lazio, Sardinia, and Lombardy. The sampled horses belonged to different breeds: 27 Thoroughbred horses, 53 Italian Standardbred horses, six Arabian horses, three Italian Saddle horses, three Quarter Horses, and two Shires.

Samples were stored at -20 °C in 2 mL tubes containing 1000 µL of DNA/RNA Shield Stabilization Solution (Zymo Research, CA, USA) until processing. Total viral nucleic acid extraction was performed using a QIAamp DNA kit (Qiagen, Milan, Italy) with a QIAcube instrument (Qiagen, Milan, Italy), according to the manufacturer's instructions. For each sample, total DNA was extracted from 200 µL of DNA/RNA Shield Stabilization Solution (Zymo Research, CA, USA). Samples were eluted in 100 µL of elution buffer (Qiagen, Milan, Italy), and DNA concentration was measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

ddPCR

For ddPCR (Bio-Rad Laboratories, Hercules, CA, USA), a QX200 ddPCR system was used according to the manufacturer's instructions. The reaction was performed in a final volume of 22 µL, consisting of 11 µL of ddPCR Supermix for Probes (2X; Bio-Rad Laboratories, Hercules, CA, USA), 0.9 µM of primer, and 0.25 µM of probe, along with 7 µL of sample DNA corresponding to 100 ng.

The primer and probe sequences for OaPV (Table 1), the tool used to generate droplets, and the thermal profiles have been described previously[8,12]. The droplets were detected using a Bio-Rad QX200 Droplet Reader. Data were analyzed to determine the OaPV types and copy numbers using QuantaSoft software version 1.4 (Bio-

Rad). Manual thresholds were applied to both OaPV genotypes and positive controls. The positive controls for OaPV1 and OaPV2 were artificially created plasmids (vector: pUCIDT-AMP) containing 270 and 603 base pairs of the sequence of E5 and the major capsid protein, respectively (IDT, Integrated DNA Technologies, IA, USA). The positive control for OaPV3 was a plasmid (vector: pUC19) containing the complete genome of OaPV3, and the positive control tissue for OaPV4 was a cutaneous fibropapillomatosis sample, both from the Department of Veterinary Medicine of Sassari University (a kind gift from Prof. A. Alberti). An OaPV-negative sample and a non-template control were included in each run. The OaPV concentration was expressed as the number of DNA copies/µL. The PCR result could be directly converted into copies/µL in the initial samples by multiplying it with the total volume of the reaction mixture (22 µL) and subsequently dividing that number by the volume of DNA sample added to the reaction mixture (7 µL) at the beginning of the assay. Each sample was analyzed in duplicate to ensure accuracy. Samples were considered OaPV-positive if at least three droplets containing OaPV amplicons were present, as suggested for PV infections in humans and veterinary medicine[9,11,27]. Furthermore, samples with fewer than 20 positive droplets were reanalyzed to ensure that these low-copy-number samples were not caused by cross-contamination.

qPCR

The qPCR assays were performed on the CFX96 Real-Time System of the C1000 Touch™ Thermal Cycler (Bio-Rad), using 96-well plates (Hard-Shell® 96-Well PCR Plates, #hsp9601; Bio-Rad). The following thermal cycling program was used: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 58 °C for 60 s. The reaction mixture (final volume 20 µL) contained 1 × TaqMan Universal Master Mix (Applied Biosystems), 900 nM each of forward and reverse primers, 250 nM of the probe (Table 1), and 100 ng of DNA sample. Each sample was amplified in duplicate. Data acquisition and analysis were performed using CFX Maestro™ (Bio-Rad). Ct values were determined using regression analysis, and samples with no Ct values were considered negative. The same samples used as positive controls for ddPCR were also tested using qPCR.

Statistical analysis

The difference in prevalence detected by ddPCR and qPCR was tested using McNemar's test. All statistical analyses were performed using the STATA Statistical Software version 17 (STATACorp, College Station, Texas, USA) (www.stata.com). Additionally, among the identified positive samples, Fisher's exact test (two-sided) was performed using Prism version 10 (GraphPad Software Inc., San Diego, CA, USA) to compare the results obtained using ddPCR and qPCR. Statistical significance was set at $p \leq 0.05$.

Data availability

The data supporting the findings of this study are available from the corresponding author upon request.

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Author contributions
S.R. was the primary investigator in this study. A.C., E.R., F.S., N.Z.,: acquisition and analysis of the data; G.P., L.L.,: visualization and statistical analysis; S.R.: conceptualization, supervision, writing (original draft), writing (review & editing). All authors reviewed the results; they approved the final version of the manuscript and declared that the content has not been published elsewhere.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
Vaginal swabs were performed in horses admitted to the Veterinary Teaching Hospitals of Turin (OVU) for causes not related to pathologies of the genital system. All animal studies were approved by the Institutional Ethics Committee of Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta (approval number 14047 of 11/28/2019). Permission to collect vaginal swabs was obtained from the animals' owners who were previously informed and in agreement with the purpose and methods used.

Additional information
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