

# Detection and quantification of bovine papillomavirus DNA by digital droplet PCR in sheep blood

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## Abstract

Abstract – Highly pathogenic bovine Delta papillomaviruses ( $\delta$ PVs) were detected and quantified for the first time using digital droplet polymerase chain reaction (ddPCR) by liquid biopsy in 103 clinically healthy sheep. Overall, ddPCR detected bovine  $\delta$ PVs in 68 blood samples (66%). Bovine papillomavirus (BPV) infection by a single genotype was revealed in 59% of the blood samples, and BPV coinfection by double, triple or quadruple genotypes was observed in 41% of liquid biopsies. The BPV-2 genotype was most frequently seen in sheep, whereas BPV-1 was the least common. Furthermore, ddPCR was very useful for detection and quantification; the BPV-14 genotype was observed for the first time in ovine species, displaying the highest prevalence in some geographical areas (Apulia). In 42 of the positive samples (61.8%), a single BPV infection was observed, 26 of which were caused by BPV-2 (61.9%) and 7 by BPV-13 (16.7%). BPV-14 was responsible for 7 single infections (16.7%) and BPV-1 for 2 single infections (4.7%). Multiple BPV coinfections were observed in the remaining 26 positive samples (38.2%), with dual BPV-2/BPV-13 infection being the most prevalent (84.6%). BPV infection by triple and quadruple genotypes was also observed in 11.5% and 3.8% of cases, respectively. The present study showed that ddPCR, a biotechnological refinement of conventional PCR, is by far the most sensitive and accurate assay for BPV detection. Therefore, ddPCR displayed diagnostic and epidemiological value resulting in the identification of otherwise undetectable BPV genotypes as well as their geographical distributions and suggesting that animal husbandry practices contribute to cross-species transmission of BPVs.

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Running title: BPV DNA by ddPCR in sheep blood

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**Abstract** – Highly pathogenic bovine *Deltapapillomaviruses* ( $\delta$ PPVs) were detected and quantified for the first time using digital droplet polymerase chain reaction (ddPCR) by liquid biopsy in 103 clinically healthy sheep. Overall, ddPCR detected bovine  $\delta$ PPVs in 68 blood samples (66%). Bovine papillomavirus (BPV) infection by a single genotype was revealed in 59% of the blood samples, and BPV coinfection by double, triple or quadruple genotypes was observed in 41% of liquid biopsies. The BPV-2 genotype was most frequently seen in sheep, whereas BPV-1 was the least common. Furthermore, ddPCR was very useful for detection and quantification; the BPV-14 genotype was observed for the first time in ovine species, displaying the highest prevalence in some geographical areas (Apulia). In 42 of the positive samples (61.8%), a single BPV infection was observed, 26 of which were caused by BPV-2 (61.9%) and 7 by BPV-13 (16.7%). BPV-14 was responsible for 7 single infections (16.7%) and BPV-1 for 2 single infections (4.7%). Multiple BPV coinfections were observed in the remaining 26 positive samples (38.2%), with dual BPV-2/BPV-13 infection being the most prevalent (84.6%). BPV infection by triple and quadruple genotypes was also observed in 11.5% and 3.8% of cases, respectively. The present study showed that ddPCR, a biotechnological refinement of conventional PCR, is by far the most sensitive and accurate assay for BPV detection. Therefore, ddPCR displayed diagnostic and epidemiological value resulting in the identification of otherwise undetectable BPV genotypes as well as their geographical distributions and suggesting that animal husbandry practices contribute to cross-species transmission of BPVs.

**Keywords:** bovine papillomavirus (BPV) DNA; digital droplet PCR( ddPCR); liquid biopsy; peripheral blood; viral load.

## 1. Introduction

Papillomaviruses (PVs) comprise a diverse group of epitheliotropic, double-stranded DNA viruses that infect humans and animals in a species-specific manner (IARC, 2007). PVs have co-evolved with their respective hosts, resulting in minimal cross-transfer between species (Doorbar et al., 2016). Viruses such as PVs that slowly evolve with their hosts typically cause latent infection (Antonsson and McMillan, 2006). However, persistent infection by PVs causes a variety of severe diseases, including cutaneous and mucosal cellular proliferation (IARC, 2007).

Bovine papillomaviruses (BPVs) comprise 29 genotypes (<http://pave.niaid.nih.gov/>; Yamashita-Kawanishi et al., 2020). Four highly pathogenic BPVs (BPV-1, -2, -13, and -14) belong to the *Delta* genus ( $\delta$ PPVs). They are associated with both cutaneous and mucosal benign and malignant tumors. Indeed, BPV-2 and BPV-13 are the most notable infectious agents commonly responsible for bladder tumors in some breeds of pasture-residing cattle that graze on lands rich in bracken fern (*Pteridium* spp.) (Campo et al., 1992; Borzacchiello et al., 2003).

$\delta$ PPVs are the only BPVs known to infect mesenchymal tissues and to show cross-species transmission and infection (IARC, 2007).  $\delta$ PPVs have been detected in cutaneous wart lesions from ovines (Mazzucchelli-de-Souza et al., 2018; Savini et al., 2020). Vertical transmission of  $\delta$ PPVs in sheep, resulting in oral fibropapillomatosis and epidermal hyperplasia of the lip in newborn lambs, has also been documented (Roperto et al., 2018a). Furthermore,  $\delta$ PPV DNA has been detected by polymerase chain reaction (PCR) in the peripheral blood of healthy sheep (Roperto et al., 2018b).

Although there are very limited numbers of reports describing BPV quantification data, PV studies have traditionally used real-time quantitative PCR (RT-qPCR) to measure the virus reservoir represented by PV DNA in both cutaneous and bladder neoplastic samples (Yuan et al., 2007; Pangty et al., 2010; Pathania et al., 2012; Cota et al., 2015). Most recently, digital PCR is gaining popularity as a novel approach to nucleic acid quantification as it allows for absolute target quantification. Indeed, digital droplet PCR (ddPCR) is a robust PCR technique that enables precise and accurate absolute quantification of target molecules by diluting and partitioning the samples into numerous compartments (Kanagal-Shamanna, 2016).

Quantification of PVs by digital PCR is proving to be a valuable improvement over qPCR, as it has been

shown to have a higher robustness to mismatches between the primer-probe set and PV genotypes. Due to pathogens that cause latent infection, BPV concentrations in the blood are sometimes too low to be determined by traditional methods. In cattle and goats, ddPCR has been found to outperform RT-qPCR in terms of the sensitivity, specificity, and reproducibility of BPV detection, all of which play a central role in diagnostic and epidemiological procedures to identify the geolocalization of BPVs (De Falco et al., 2020; Cutarelli et al., submitted).

The present study aimed to detect and quantify BPV DNA in the peripheral blood of sheep using ddPCR and to show the potential advantages of this molecular technology in the diagnosis and epidemiology of infectious diseases, including viral diseases.

## 2. Materials and Methods

### 2.1 Ethics statement

Blood samples were collected from animals in public slaughterhouses during the mandatory ante-mortem clinical examination. All procedures performed in this study followed common good clinical practices and received institutional approval from the Ethical Animal Care and Use Committee of the University of Naples Federico II (PG/2017/ 0099607). All farmers were previously informed and in agreement with the purpose and methods used.

### 2.2 Liquid biopsy samples and DNA extraction

Blood samples from 103 apparently healthy 1- to 3-year-old sheep were collected from the jugular vein in vacutainers containing ethylenediaminetetraacetic acid (EDTA). A total of 40 samples were obtained from sheep living in Sardinia (Sar) (20) and Campania (Cam) (20), 48 samples from Calabria (Cal) (24) and Basilicata (Bas) (24), and 15 samples from Apulia (Apu). All sheep, excluding those from Cam, were from flocks that lived and shared the bracken fern-infested lands that they grazed on with pasture-residing cattle. Sheep from Cam were from flocks living in closed pens without any contact with other animals. Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Wilmington, DE, USA), according to the manufacturer's instructions.

### 2.3. RT-qPCR

RT-qPCR was performed in a final volume of 20  $\mu$ L containing 10  $\mu$ L of TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of each of the forward and reverse primers (Bio-Rad Laboratories, Hercules, CA, USA), 250 nM of the probe (Bio-Rad Laboratories), and 100 ng of the DNA sample. The primers and probes for the detection of four BPV genotypes (BPV-1, -2, -13, and -14) were used as reported elsewhere (De Falco et al., 2020). The reaction was performed on the CFX96 Real-Time System of the C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories). The thermal cycling conditions were as follows: 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 (acquiring FAM and VIC dyes). Each sample was analyzed in duplicate, and negative controls were included in all runs. Data acquisition and data analyses were performed using CFX Maestro™ (Bio-Rad Laboratories) software. The same samples used as positive controls for ddPCR were also tested using RT-qPCR.

### 2.4. DdPCR

For ddPCR, Bio-Rad's QX100 ddPCR System was used according to the manufacturer's instructions. The reaction was performed in a final volume of 20  $\mu$ L containing 10  $\mu$ L of ddPCR Supermix for Probes (no dUTP 2 $\times$ ; Bio-Rad), 0.9  $\mu$ M primer, and 0.25  $\mu$ M probe together with 5  $\mu$ L sample DNA (100 ng). A black hole quencher was used in combination with FAM and VIC fluorescent dye reporters (Bio-Rad Laboratories). The reaction mixture was placed into the sample well of a DG8 cartridge (Bio-Rad Laboratories). A volume of 70  $\mu$ L of droplet generation oil was loaded into the oil well, and droplets were formed in the droplet generator (Bio-Rad Laboratories). After processing, the droplets were transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany). PCR amplification was carried out on a T100 Thermal Cycler (Bio-Rad Laboratories) with the following thermal profile: hold at 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 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) and the droplets from each well of the plate were read automatically. QuantaSoft software was used to count the PCR-positive and PCR-negative droplets to provide absolute quantification of the target DNA. Therefore, the ddPCR results could be directly converted into copies/ $\mu\text{L}$  in the initial samples simply by multiplying them by the total volume of the reaction mixture (20  $\mu\text{L}$ ) and then dividing that number by the volume of DNA sample added to the reaction mixture (5  $\mu\text{L}$ ) at the beginning of the assay. Samples with very few positive droplets were re-analyzed to ensure that these low copy number samples were not due to cross-contamination.

## 2.5 Statistical analysis

Differences in the proportions of detected cases were tested using the chi-square test by Campbell and Richardson (Richardson, 2011). Furthermore, regarding the significance relative to the number of copies of BPV DNA detected in sheep in the different regions, the t-test was used after adjusting for the Bonferroni multiple comparison correction of means. P-values  $[?] .05$  were considered to be statistically significant. All analyses were performed using R statistical software (The R Foundation, Vienna, Austria).

## 3. Results

Overall, our results showed that BPV DNA was found in 68 out of 103 blood samples (66%) from healthy sheep using ddPCR. The same liquid biopsies were also investigated using RT-qPCR, which revealed BPV DNA in approximately 9% of blood samples (Fig 1). In 42 of the positive samples (61.8%), a single BPV infection was observed (Fig. 2), 26 of which were caused by BPV-2 (61.9%) and 7 by BPV-13 (16.7%). BPV-14 was responsible for 7 single infections (16.7%), and BPV-1 for two single infections (4.7%) (Fig. 3). Multiple BPV infections were seen in 26 (38.2%) positive samples. BPV coinfections caused by two genotypes were seen in 22 positive cases (84.6%), with dual BPV-2/BPV-13 infection being the most prevalent. BPV coinfections by triple and quadruple genotypes were detected in 11.5% (3/26) and 3.8% (1/26) of blood samples, respectively (Fig. 4). In sheep flocks that lived and shared lands with cattle, BPV DNA was detected in approximately 53% of blood samples collected in Apu (8/15), 75% of samples acquired in both Bas and Cal (18/24), and 100% of blood samples harvested from Sar (20/20). In sheep flocks from Cam that lived in isolated and closed pens without any contact with cattle, BPV DNA was detected in 20% of blood samples examined (4/20). The percentage differences in BPV infections in all sheep flocks with cattle contact were statistically significant compared to the percentage observed in sheep flocks without any contact with cattle, as the Campbell-Ricardson's chi-square test resulted in a p-value  $< 0.05$ . Furthermore, in all geographical areas except for Apu, BPV-2 was the most prevalent genotype. BPV-13 and BPV-14, as well as BPV-1 were also observed. Furthermore, Apu BPV-14 showed very high numbers of copies/ $\mu\text{L}$  (mean value of 895.2); it was the most prevalent BPV genotype at a detection level of 40% in the examined samples (6/15) and a statistically significant Campbell-Ricardson's chi-square test p-value  $< .05$ .

The overall quantification results showed that viral copy numbers/ $\mu\text{L}$  ranged from 76 to 568 for BPV-1 (mean value = 183.6 ), 65 to 3768 for BPV-2 (mean value = 397.1), 66 to 2112 for BPV-13 (246.5), and 76 to 1768 for BPV-14 (mean value = 447.5). When sheep flocks from Sar alone were considered, BPV-2 showed the highest copy numbers/ $\mu\text{L}$  with a mean of 1162. Using t-tests, the differences between the copy numbers of BPV-2 in Sar compared to the other means found in Cal, Cam, Bas, and Apu were statistically significant with p-values  $< .05$ . Indeed, after adjusting for the Bonferroni multiple comparison correction, their p-values were .003 (Sar-Cal), .04 (Sar-Cam), .002 (Sar-Bas), and .01 (Sar-Apu), respectively. Table 1 summarizes the quantitative data using QuantaSoft software, demonstrating the numbers of copies/ $\mu\text{L}$  in blood samples from sheep flocks located in the five regions in South Italy.

## 4. Discussion

To our knowledge, this study is the first to use the sensitive ddPCR assay, a biotechnological refinement of conventional PCR, to detect and quantify highly pathogenic bovine  $\delta\text{PVs}$  in clinically healthy sheep by liquid biopsy. Our previous studies have shown that ddPCR has both high specificity and sensitivity for the detection and quantification of BPV DNA in healthy and diseased cattle (De Falco et al., 2020) as well

as in healthy goats (Cutarelli et al., submitted). The current study provides further evidence that ddPCR is a very useful approach to detect and quantify BPV in the blood of healthy sheep and allows us to gain diagnostic and epidemiological insights into BPV presence in ovine species as data on the prevalence and types of BPVs in sheep are not currently available.

The liquid biopsy approach to the detection of circulating BPV DNA has garnered growing interest in PV studies (Veyer et al., 2020). Indeed, PV detection in the blood can be used as diagnostic, prognostic, and epidemiological markers (Bishop et al., 2016).

Our study showed that BPV-2 is the most prevalent BPV genotype in healthy sheep, similar to other ruminants, such as cattle and goats. The highest number of copies of this genotype was found in Sar from sheep flocks in which cross-infections by BPVs have been previously reported (Roperto et al., 2018a), which suggests that copy numbers may correlate with the risk of cutaneous and mucosal lesions that progress to cancer. Furthermore, this study reports the first detection of BPV-14 in sheep. This genotype, chronologically the last BPV type identified in cattle, has never been described in the ovine species. Furthermore, our results demonstrated a statistically significant prevalence of BPV-14 in Apu compared to Sar, Cal, Bas, and Cam, which clearly showed that BPV genotype prevalence has a territorial divergence in these regions.

We compared the sensitivity of ddPCR with that of RT-qPCR in evaluating the same liquid biopsy, demonstrating that ddPCR has superior sensitivity compared to RT-qPCR. Therefore, our results suggest that ddPCR is by far the most sensitive and accurate assay for BPV detection. It is worth noting that it has been shown that ddPCR outperforms RT-qPCR in terms of the sensitivity, specificity, and reproducibility of oncogenic human papillomavirus detection and quantification (Biron et al., 2016; Jeannot et al., 2016; Carow et al., 2017).

BPV genotype detection in the blood of healthy sheep suggests that the bloodstream can be the primary site of BPV infection. As bovine  $\delta$ PVs are known to infect ovine species and result in anatomoclinical diseases, it is conceivable that these viruses may spread through the blood, which could be responsible for secondary tissue localization and infection. However, as in humans (IARC, 2007), further epidemiological studies are required to enhance the understanding of BPV transmission via the bloodstream.

Here, we detected a higher percentage of BPVs in sheep that were in close contact with cattle herds, about which numerous case reports of BPV infection have been described. Indeed, the sheep flocks in our study shared bracken fern-infested lands with pasture-residing cattle for grazing. The evidence from epidemiological studies of cattle is sufficiently strong to suggest that in the presence of BPV infection, the toxic components of bracken ferns such as ptaquiloside (PT), a water-soluble norsesquiterpenoid glycoside, are ecological co-factors in the development of severe diseases due to BPVs, including chronic enzootic hematuria (CEH), a clinical syndrome caused by bladder tumors (Smith, 1990). Thus, it is conceivable that PT may also be a co-factor of diseases in sheep. Indeed, PT has recently been detected in biological matrices from healthy sheep (Virgilio et al., 2015). PT is known to hamper the immune system and may play an important role in cross-species transmission and infection of bovine  $\delta$ PVs. It is worth noting that outbreaks of CEH have also been reported in sheep (Prakash et al., 1996; Cianciolo and Mohr, 2016). Furthermore, bovine  $\delta$ PV infection resulting in clinical disease are known to occur in sheep (Mazzucchelli-de-Souza et al., 2018; Roperto et al., 2018a; Savini et al., 2020). Therefore, the detection of bovine  $\delta$ PV DNA in the blood of sheep means that sheep can be infected by these PVs, which may make  $\delta$ PVs an additional, potential cause of ovine disease. Furthermore, our results suggest that clinically healthy sheep may represent a reservoir for bovine  $\delta$ PVs. Thus, it is conceivable that sheep may play a role in intra- and interspecies bovine  $\delta$ PV transmission and infection. In this context, very precise quantitation of very low viral copy numbers can provide more precise monitoring of latent BPV DNA reservoirs.

Finally, it is well known that PV distribution varies considerably by geography (Chen et al., 2019). Therefore, ddPCR may be an essential tool for improving diagnostic procedures thus allowing the identification of the genotypic distribution of BPV and a better understanding of the possible, geographical divergence of BPV prevalence in different areas. The ddPCR assay appears to possess high sensitivity and accuracy, which is

valuable for addressing the molecular burden of BPV infections and useful for defining an accurate ecological epidemiology. This baseline information improves our knowledge about the molecular mechanisms of the disease and provides insights into necessary measures for reducing the risk of BPV infection and/or co-infection.

In conclusion, ddPCR is presently being used to detect very low nucleic acid concentrations and, therefore, appears to be of interest in the diagnosis of infectious diseases, including viral diseases (Li et al., 2018). DdPCR has proven to be a valuable new technology and with additional improvements in prospect it is likely to become an indispensable tool in diagnostic, prognostic and epidemiological virus research (Rutsaert et al. 2018). Therefore, the ddPCR method may provide a new and promising tool for evaluating the BPV viral load in clinical samples. Future PV research warrants the use of this molecular approach to assess PV type-specific pathogenetic pathways of disease, including carcinogenicity. Indeed, available evidence from BPV distribution lends strong support to the notion that the risk of an animal developing a BPV-associated disease varies substantially according to the specific BPV type with which the animal is infected. Finally, the ddPCR approach may provide a better understanding of the complex interactions between multiple BPV types during coinfections, as the possible interference resulting from multiple PV genotypes in coinfection cases remains an open question (Biryukov and Meyers, 2018).

### Declaration of conflicting interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Figure legends

Figure 1. Percentage of circulating BPVs detected by ddPCR and RT-qPCR in blood samples from healthy sheep.

Figure 2. Detection of circulating BPVs. Single and multiple BPV genotypes were detected.

Figure 3. Overall percentage of circulating BPVs by single genotypes. BPV2 was by far the most prevalent genotype detected in all geographical regions.

Figure 4. BPV coinfection by double genotypes (A) represented the majority of virus infections by multiple types. Coinfections by triple (B) and quadruple (C) genotypes were rarely seen.

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