The diagnostic value of the droplet digital PCR for the detection of bovine Deltapapillomavirus in goats by liquid biopsy

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Abstract

In the present study, the highly pathogenic bovine Deltapapillomavirus (δPV) was investigated by liquid biopsy in blood samples of 168 clinically normal goats using both droplet digital PCR (ddPCR) and quantitative real time PCR (qPCR). Overall, ddPCR detected BPV E5 DNA in ˜61.3% of the blood samples examined, while real time qPCR revealed the virus in ˜10.7% of the same samples. Moreover, ddPCR showed BPV E5 DNA in ˜78.8% of blood samples from goats that were in close contact with cattle and in 20% of blood samples from goats living in closed pens without any contact with cattle. In addition, ddPCR revealed a single BPV genotype in ˜59.2% and multiple genotypes in ˜40.8% of goats harboring BPV DNA, while real time qPCR detected single genotypes in ˜17% and multiple genotypes in ˜1%. Of the BPV co-infections detected by ddPCR, 28 (˜67%) involved two genotypes, eight (˜19%) three genotypes, and six (˜14%) four genotypes. In contrast, real time qPCR revealed BPV co-infection by two genotypes in only one sample and failed to detect co-infection by three or four genotypes. BPV2 and BPV13 were the most prevalent viruses responsible for single and multiple co-infections, respectively. The present study showed that the ddPCR technique has much higher sensitivity and specificity in the detection of these viruses, and suggested that animal husbandry practices contribute to cross-species transmission of BPVs.

The diagnostic value of the droplet digital PCR for the detection of bovine *Deltapapillomavirus* in goats by liquid biopsy

Running title: DdPCR approach for the detection of circulating bovine papillomavirus

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ABSTRACT – In the present study, the highly pathogenic bovine Deltapapillomavirus (δPV) was investigated by liquid biopsy in blood samples of 168 clinically normal goats using both droplet digital PCR (ddPCR) and quantitative real time PCR (qPCR). Overall, ddPCR detected BPV E5 DNA in ˜61.3% of the blood samples examined, while real time qPCR revealed the virus in ˜10.7% of the same samples. Moreover, ddPCR showed BPV E5 DNA in ˜78.8% of blood samples from goats that were in close contact with cattle and in 20% of blood samples from goats living in closed pens without any contact with cattle. In addition, ddPCR revealed a single BPV genotype in ˜59.2% and multiple genotypes in ˜40.8% of goats harboring BPV DNA, while real time qPCR detected single genotypes in ˜17% and multiple genotypes in ˜1%. Of the BPV co-infections detected by ddPCR, 28 (˜67%) involved two genotypes, eight (˜19%) three genotypes, and six (˜14%) four genotypes. In contrast, real time qPCR revealed BPV co-infection by two genotypes in only one sample and failed to detect co-infection by three or four genotypes. BPV2 and BPV13 were the most prevalent viruses responsible for single and multiple co-infections, respectively. The present study showed that the ddPCR technique has much higher sensitivity and specificity in the detection of these viruses, and suggested that animal husbandry practices contribute to cross-species transmission of BPVs.

Keywords: blood; bovine papillomavirus (BPV); droplet digital polymerase chain reaction (ddPCR); goats; quantitative real time polymerase chain reaction (qPCR).

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1. Introduction

The bovine papillomaviruses (BPVs) comprise 29 genotypes within five genera: *Deltapapillomavirus* (δPV**;** BPV-1, -2, -13, and -14), *Xipapillomavirus* (χPV; BPV-3, -4, -6, -9, -10, -11, -12, -15, -17, -20, -23, -24, -26, -28, and 29), *Epsilonpapillomavirus*(εPV; BPV-5, -8, and -25), *Dyokappapapillomavirus* (Dyoκ PV; BPV-16, -18, -22), *Dyoxipapillomavirus* (Dyoχ PV; BPV-7). In addition, BPV-19, -21, and -27 remain to be classified. (http://pave.niaid.nih.gov/; Yamashita-Kawanishi et al., 2020a; 2020b).

Bovine δPVs recognize *Bos taurus* as their classical host. However, they are also the only papillomaviruses capable of natural cross-species transmission and infection (IARC, 2007). Indeed, beyond cattle, bovine δPVs have been extensively investigated in horses (Epperson and Castleman, 2017; Savini et al., 2019), sheep (Mazzucchelli-de-Souza et al., 2018; Roperto et al., 2018; Savini et al., 2020) and buffaloes (Somvanshi, 2011; Roperto et al., 2013). They have even been studied in wild ruminants (Savini et al., 2016).

Quantitative real time polymerase chain reaction (qPCR) is considered the assay with the highest sensitivity and specificity for detecting papillomavirus DNA (Biron et al., 2016). As such, it has long been the method of choice for estimating viral load. However, to quantify real time qPCR, standard curves are necessary, and the efficiency of the method may vary between runs and reactions (Clementi and Bagnarelli, 2015).

Droplet digital PCR (ddPCR), first described by Sykes et al. (1992), is an improved method of conventional PCR that can clonally amplify and directly quantify DNA or RNA (Li et al., 2018). The technique involves diluting and partitioning the sample in many reaction chambers or droplets. Because ddPCR can perform absolute quantitation without a well-calibrated standard or highly efficient amplification, it is useful for determining of pathogen loads being faster, more precise, and reproducible (Kuypers and Jerome, 2017). Indeed, ddPCR is currently the most accurate and sensitive method for quantifying nucleic acids of interest particularly in cases of low pathogen loads (Isaac et al., 2017). Because it is more sensitive than classical approaches, including real time qPCR, it can detect small amounts of DNA that indicate residual disease and may otherwise be undetectable. For this reason, ddPCR has been used to quantify many viruses being especially useful for precise quantification of low viral loads (Kuypers and Jerome, 2017). In particular, precise quantitation of very low viral copy numbers may permit more reliable monitoring of latent papillomavirus DNA reservoirs, allowing early diagnosis and more timely treatment of viral infections (Li et al., 2018). Furthermore, ddPCR yields significantly fewer inaccurate false negatives and positives than real time qPCR in samples with low viral load (Liu et al., 2020).

The aim of the present study was to investigate, not previously reported, viral load of bovine δPVs in blood

from clinically normal goats, as well as to compare ddPCR with real time qPCR to evaluate the diagnostic value of the ddPCR assay for the detection and quantification of BPVs by liquid biopsy.

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 agreed with the purpose and methods used.

2.2 Blood samples and DNA extraction

Blood samples from 168 goats were obtained from the jugular vein and collected in vacutainers containing EDTA. Among these goats, 118 lived in close contact with cattle on lands rich in bracken fern (*Pteridium* spp.), which they grazed on. Cattle who share pastures with goats tend to develop chronic enzootic hematuria, a clinical syndrome caused by papillomavirus-associated bladder tumors. The remaining 50 goats were from flocks living in closed pens. They were fed fern-free hay and had no contact with cattle. Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Wilmington, DE, USA), according to the manufacturer's instructions.

2.3 Digital droplet polymerase chain reaction

The primers and probes for the detecting four BPV genotypes (BPV-1, -2, -13, and -14) were used as reported elsewhere (De Falco et al., 2020). A black hole quencher was used together with the fluorescent reporter dyes FAM and VIC. The ddPCR assay mixture contained 10 µL of 2×ddPCR Supermix for Probes (Bio-Rad, Munich, Germany), 900 nmol/L of each primer, 250 nmol/L of probes, 5 µL of the DNA sample (100 ng), and enough sterile distilled water to ensure a final volume of 20 µL. Droplets were generated using a QX100 droplet generator (Bio-Rad, Munich, Germany), according to the manufacturer's instructions. The droplets (˜40 µL) were transferred to a 96-well plate, which was then heat-sealed. The PCR amplification was performed in a T100 Touch thermal cycler (Bio-Rad, Munich, Germany) using the following conditions: 95°C for 10 min; 40 cycles of 94°C for 30 s, 56°C for 1 min, and 98°C for 10 min; final hold at 12°C. Next, the droplets were analyzed using a QX100 droplet reader (Bio-Rad, Munich, Germany). Data were analyzed using QuantaSoft software 1.4 (Bio-Rad, Munich, Germany). All tests were repeated twice. Manual thresholds were applied to both the BPV genotypes and the positive controls, according to our previous research (De Falco et al., 2020). In each run, a BPV-negative sample and a non-template control were included. The BPV concentration was expressed as the number of copies of DNA per microliter of blood (copies/µL). Therefore, the PCR result could be directly converted into copies/µL in the initial samples simply by multiplying it with the total volume of the reaction mixture (20 µL) and dividing the result by the volume of DNA sample added to the reaction mixture at the beginning of the assay (5 µL).

2.4 Quantitative real-time polymerase chain reaction

The qPCR assays were performed on the CFX96 Real-Time System of the C1000 Touch^{TM} Thermal Cycler (Bio-Rad), using 96-well plates (Hard-Shell® 96-Well PCR Plates, #hsp9601; Bio-Rad). The final PCR volume was 20 µL containing: 1x TaqMan Universal Master Mix (Applied Biosystems), 900 nM each of the forward and reverse primers, 250 nM of the probe, and 100 ng of the DNA sample. The following thermal cycling program was used: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 58°C for 60 s. Each sample was analyzed in duplicate, and the Ct values were determined using regression analysis. Data acquisition and data analyses were performed using CFX Maestro^{TM}(Bio-Rad) software. The same samples used as positive controls for ddPCR were also tested using real time qPCR.

2.5 Statistical analysis

Differences in the proportions of cases detected were tested using the χ^2 test of Campbell and Richardson (Richardson, 2011), with p-values [?] .05 indicating statistical significance.

3. Results

Overall, ddPCR detected BPV DNA in ˜61.3% (103/168) of the blood samples examined, while real time qPCR revealed the virus in ˜10.7% (18/168) of the same samples (p [?] .001). Moreover, ddPCR showed BPV DNA in ˜78.8% (93/118) of blood samples from goats that were in close contact with cattle and in 20% (10/50) of blood samples from goats living in closed pens without any contact with cattle (p [?] .001), suggesting that animal husbandry practices contribute to virus spread and, very likely, to cross-species transmission of BPVs. In addition, ddPCR revealed a single BPV genotype in ˜59.2% (61/103) and multiple genotypes in ˜40.8% (42/103) of the goats harboring BPV DNA, while real time qPCR detected single genotypes in ˜17% (17/103) and multiple genotypes in ˜1% (1/103). Furthermore, real time qPCR failed to detect BPV DNA in samples tested negative by ddPCR. BPV2 and BPV13 were the most frequent genotypes detected in single infections, while only occasionally were BPV1 and BPV14 found alone. Of the BPV co-infections detected by ddPCR, 28 (˜67%) involved two genotypes, eight (˜19%) three genotypes, and six (˜14%) four genotypes. In contrast, real time qPCR revealed BPV co-infection by two genotypes in only one sample and failed to detect co-infection by three and four genotypes. Co-infections by BPV2 and BPV13 were the most frequent infections. Table 1 summarizes these results, while Table 2 shows the BPV genotype frequencies. Furthermore, the ddPCR assay was also carried out for quantification of BPV, with the results showing remarkable distinctions between positive FAM (blue), and VIC (gree) and negative (grey) droplets (Figure 1-4). There were also differences in the fluorescence amplitude range of the background (negative) droplets among the BPV samples, that is 3,000-9,000 for BPV-1, 4,000-16,000 for BPV-2, 4,000-16,000 for BPV-13 and 2,000-6,000 for BPV-14. Table 3 reports the numbers of BPV DNA copies per microliter of blood samples from 168 clinically healthy goats.

4. Discussion

To our knowledge, this study was the first to detect and quantify highly pathogenic bovine δPVs from clinically normal goats using the sensitive ddPCR approach. We previously showed that this approach has the highest sensitivity and specificity for detecting and quantifying bovine δPVs in cattle (De Falco et al., 2020).

Overall, ddPCR detected BPV E5 DNA in a greater percentage of the blood samples than real time qPCR, and ddPCR quantified the number of E5 DNA copies per microliter of blood for all four bovine δPV genotypes. In particular, BPV genotype detection was significantly more common in goats That lived in close contact with cattle suffering from anatomoclinical δPV-related pathology than in those from closed pens that had no contact with cattle. This suggests that cattle are the major source of bovine δPVs in goats. Furthermore, the goats harboring a higher prevalence of δPVs in the blood lived with cattle on lands contaminated by bracken fern (*Pteridium aquilinum*), so they may have ingested this plant. The major toxic substance of bracken fern is ptaquiloside, which impairs the immune system and has recently been detected in healthy goats (Virgilio et al., 2015). Therefore, bracken fern may also facilitate cross-species transmission of δPVs from cattle to goats.

We compared diagnostic sensitivity between ddPCR and real time qPCR by using them to evaluate the same liquid biopsy. The results showed that ddPCR had superior sensitivity to real time qPCR, which is believed to be the gold standard for measuring papillomavirus DNA (Isaac et al., 2017). All differences in the detection of circulating BPV E5 DNA were significant, confirming that ddPCR is a more reliable approach. Therefore, ddPCR should confer markedly improved diagnosis of BPV infection over both PCR and real time qPCR. The present study clearly demonstrated that ddPCR outperforms real time qPCR in terms of the sensitivity, specificity, and reproducibility of BPV detection, similar to recent studies that focused on oncogenic human papillomavirus (HPV) detection (Biron et al., 2016; Carow et al., 2017). Therefore, as for human samples (Cheung et al., 2019), ddPCR could be a diagnostic procedure capable of detecting otherwise undetectable BPVs.

Our study raises important questions that warrant further investigation. Outbreaks of cutaneous and bladder diseases associated with bovine δPVs occur in small ruminants such as sheep (Mazzucchelli-de-Souza et al., 2018; Roperto et al., 2018; Savini et al., 2020). No clinical manifestations of bovine δPV infections have hitherto occurred in goats. Therefore, it is unlikely that bovine δPVs can cause infections in goats that result in clinical symptoms. However, the present study suggested that goats play a major role in the epidemiology of the virus, as they may serve as an environmental reservoir. Indeed, bovine δPVs were found in goats living in close proximity with cattle more than in goats that had no contact with cattle.

It may be that ddPCR is approximately 500 times more sensitive than real time qPCR to detect low level analyte (Suo et al., 2020). As such, ddPCR can detect clinically relevant changes in viral load and could provide valuable insight to inform the development prophylactic measures and/or improve treatment outcomes. Very precise quantitation of very low viral copy numbers allows latent BPV DNA reservoirs to be monitored. Therefore, ddPCR will allow researchers to better understand BPV epidemiology. To develop appropriate prophylactic and/or therapeutic tool, as well as to determine the interspecies transmission potential and evolution of BPVs, the BPV prevalence, distribution, and clinical consequences must be identified in different animal species (Dogan et al., 2018). We believe that this type of study is remarkable for bovine δPVs, which are the only BPVs responsible for cross-species transfection and infection (IARC, 2007).

In conclusion, the ddPCR technique allows low-abundance nucleic acid detection and is useful in the diagnosis of infectious diseases. The method has excellent precision and well defined accuracy in the quantitation of ddPCR making it useful for detecting low pathogen loads. The sensitivity and specificity of ddPCR are superior to those of real time qPCR in the clinical diagnosis of infectious diseases, including viral, bacterial diseases and parasitic infections. As such, ddPCR may be a better choice for clinical and epidemiological applications in the future. DdPCR is a valuable and reliable new technology with additional improvements in prospect, it is likely to become a useful tool in future BPV research. Therefore, ddPCR could be used to improve diagnostic procedures, accurately identify the genotypic distribution of BPVs, and allow researchers to better understand the territorial divergence of BPV prevalence. Such insights will improve our understanding of the molecular and ecological epidemiology of infectious diseases, including viral ones.

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Data availability statement

All data supporting this manuscript are reported and can be found in our paper

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Figure legends

Figure 1. Digital droplet polymerase chain reaction (ddPCR) for BPV-1 detection, showing samples that were positive and negative for the E5 DNA of BPV-1. Blue droplets = positive; grey droplets = negative. QuantaSoft screenshots show the ddPCR results: C03 - D03 are weakly positive samples, D01 and F01 are positive samples, and G04 is the positive control.

Figure 2. Digital droplet polymerase chain reaction (ddPCR) for BPV-2 detection, showing samples that were positive and negative for the E5 DNA of BPV-2. Green droplets = positive; grey droplets = negative.

QuantaSoft screenshots show the ddPCR results: all samples shown are positive

Figure 3. Digital droplet polymerase chain reaction (ddPCR) for BPV-13 detection, showing samples that were positive and negative for the E5 DNA of BPV-13. Blue droplets = positive; grey droplets = negative. QuantaSoft screenshots show the ddPCR results: all samples shown are weakly positive

Figure 4. Digital droplet polymerase chain reaction (ddPCR) for BPV-14 detection, showing samples that were positive and negative for the E5 DNA of BPV-14. Blue droplets = positive; grey droplets = negative. QuantaSoft screenshots show the ddPCR results: all samples shown are weakly positive

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