FROM THE BENCH

Droplet-digital PCR assay to detect Merkel cell polyomavirus sequences in chorionic villi from spontaneous abortion affected females

Andrea Tagliapietra[1]* | John Charles Rotondo[1]* | Ilaria Bononi[1] | Elisa Mazzoni[1] | Federica Magagnoli[1] | Lucia Oton Gonzalez[1] | Carlo Contini[2] | Fortunato Vesce[3] | Mauro Tognon[1] | Fernanda Martini[1]

[1]Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, University of Ferrara, Ferrara, Italy

[2]Department of Medical Sciences, Section of Infectious Diseases and Dermatology, University of Ferrara, Ferrara, Italy

[3]Department of Morphology, Surgery and Experimental Medicine, Section of Obstetrics and Gynecology, University of Ferrara, Ferrara, Italy

Correspondence
Mauro Tognon and Fernanda Martini, Laboratories of Cell Biology and Molecular Genetics, Section of Pathology, Oncology, and Experimental Biology, School of Medicine, University of Ferrara, 64/b, Fossato di Mortara Street, 44121 Ferrara, Italy.
Email: tgm@unife.it; mrf@unife.it

Abstract

Droplet-digital polymerase chain reaction (ddPCR) technique was set up to detect/quantify Merkel cell polyomavirus (MCPyV) DNA in clinical specimens, including chorionic villi and peripheral blood mononuclear cells (PBMCs) from spontaneous abortion (SA)-affected females. This ddPCR assay showed high accuracy, sensitivity, and specificity in detecting MCPyV DNA cloned in a recombinant plasmid vector, the control. ddPCR was extended to MCPyV DNA to investigate/quantify its sequences in clinical samples. Overall, 400 samples were analyzed, that is, 100 chorionic villi and 100 PBMCs, from SA females ($n = 100$), the cases, and 100 chorionic villi and 100 PBMCs from females who underwent voluntary pregnancy interruption (VI, $n = 100$), the control. MCPyV DNA was detected in 4/100 (4%) and 5/100 (5%) of SA and VI chorionic villi, respectively. The mean viral DNA load was 1.99 (± 0.94 standard mean deviation [SD]) copy/10^4 cells in SA and 3.02 (± 1.86 [SD]) copy/10^4 cells in VI. In PBMCs, MCPyV DNA was revealed in 9/100 (9%) and 14/100 (14%) of SA and VI, with a mean of 2.09 (± 1.17 [SD]) copy/10^4 cells and 4.09 (± 4.26 [SD]) copy/10^4 cells in SA and VI, respectively. MCPyV gene expression analysis by quantitative PCR for the large T antigen (LT) and viral capsid protein 1 (VP1) showed their mRNAs in 2/4 (50%) SA- and 2/5 (40%) VI-MCPyV-positive samples. MCPyV DNA was detected/quantified using the ddPCR technique, in chorionic villi and PBMCs from SA and VI. In our experimental conditions, ddPCR provided a powerful tool to detect/quantify MCPyV DNA sequences in clinical samples.

KEYWORDS
abortion, chorionic villi, ddPCR, MCPyV, PBMC

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*Andrea Tagliapietra and John Charles Rotondo contributed equally to this study.

1 | INTRODUCTION

The pregnancy status is often affected by spontaneous abortion (SA; Contini et al., 2018; Rotondo et al., 2012; Tagliapietra et al., 2019). SA has been associated with a large number of causes, including age, ethnic origin, and lifestyle factors (de la Rochebrochard & Thonneau, 2002; Giakoumelou et al., 2016; Lashen, Fear, & Sturdee, 2004). Other causes, including endocrine, hormonal/immunological/genetic abnormalities, and male factors, have also been associated to SA (Giakoumelou et al., 2016; Rotondo et al., 2012). Infectious agents, including viruses, are responsible for spontaneous abortion cases, being the reported prevalence of SA in the range of 15–66%, depending on the study and the pregnancy period analyzed (Contini et al., 2018; Giakoumelou et al., 2016). Indeed, viruses may cause complications during pregnancy, ending, in the worst cases, in the loss of the embryo/fetus (Boldorini et al., 2010; Giakoumelou et al., 2016; Nagamori et al., 2010). The role of human polyomaviruses (HPyVs) in SA remains to be elucidated (Tagliapietra et al., 2019).

Merkel cell polyomavirus (MCPyV) is the viral agent associated with the Merkel cell carcinoma (MCC), which is a rare but aggressive tumor of the skin (Rotondo et al., 2017a). MCPyV DNA was identified in sera/buffy coats of healthy subjects (Mazzoni et al., 2017a; Pancaldi et al., 2011). In addition, MCPyV footprints were detected in uterine cervical cancer specimens (Imajoh et al., 2012), suggesting that this virus, after infecting the uterus, could participate as a risk factor in SA. The involvement of MCPyV infection in SA is poorly understood (Giakoumelou et al., 2016). Indeed, only one work investigated the putative role of MCPyV in SA, suggesting its potential cause in miscarriage (Sadeghi et al., 2010). This study employed qualitative and quantitative PCR to investigate MCPyV DNA sequences in fetal samples and aborted tissues (Sadeghi et al., 2010). However, qPCR assay shows some limitations to detect and quantify viral DNA sequences present with a low-copy number in a clinical sample, when the quantification cycle (Cq) value is near the cutoff (Caraguel, Stryhn, Gagné, Dohoo, & Hammell, 2011). In addition, the need of calibration curves, represented by recombinant plasmids carrying viral DNAs, increases the risk of false-positive results (Shah, 2006). Therefore, the development of more analytical, sensitive and accurate assays to verify the MCPyV infection in SA is needed.

The quantification of nucleic acids has been improved by the introduction of the new droplet-digital PCR (ddPCR) method. ddPCR is an emerging PCR technology, which enables to measure, in an absolute manner, viral DNA loads in clinical samples (Mazzoni et al., 2017a; Pinheiro et al., 2012; Tagliapietra et al., 2019). ddPCR technology provides a more reliable method in testing low concentrations of viral DNAs in comparison with qPCR methods (Pinheiro et al., 2012). Indeed, ddPCR provides orders of magnitude of more precision/sensitivity, as well as a higher detection rate than qPCR (Hindson et al., 2011). ddPCR, which is based on a traditional PCR reaction, works on principle of limiting dilution and Poisson statistics (Hindson et al., 2011). The limiting dilution provides the partition of the sample tested into approximately 20,000 nano-droplets. The partitioned sample is subsequently PCR amplified on a thermal cycler. Each droplet contains only one target molecule, which represents the fluorescent signal. Since each droplet is considered an independent event, ddPCR provides a single molecule sensitivity assessment thus allowing to quantify the real amount of DNA under analysis (Pinheiro et al., 2012). Indeed, droplets are discriminated in those that do not contain the target (negative) from those containing the target (positive), by the amplitude of their fluorescence signal (Hindson et al., 2011). Since the sample to be tested is randomly partitioned into droplets, the Poisson statistics is applied to calculate the copy number of the target DNA. This technical approach does not need a calibration curve, thereby detecting, in absolute manner, the DNA copy number (Pinheiro et al., 2012).

In this study, the ddPCR approach was set up to detect and quantify MCPyV DNA sequences in SA. In the first step of the analysis, ddPCR accuracy, sensitivity, and specificity were addressed to MCPyV DNA sequences cloned in a recombinant plasmid vector, employed as control. Then, the assay was extended to the MCPyV DNA target to investigate and quantify its sequences in clinical samples, including aborted tissues, such as chorionic villi, and peripheral blood mononuclear cells (PBMCs), harvested from SA affected females, the cases, and females who underwent voluntary pregnancy interruption (VI), the control. *MCPyV LT* and *VP1* gene expressions were investigated in MCPyV-positive samples.

2 | MATERIALS AND METHODS

2.1 | Sample

Clinical samples, that is, chorionic villi and PBMCs were from our biology bank (Contini et al., 2018; Tagliapietra et al., 2019). A total of 400 samples were analyzed: (a) chorionic villi (*n* = 200) and (b) PBMCs (*n* = 200) were from two different cohorts, that is, SA (*n* = 100) and VI (*n* = 100) females, the cases and control, respectively. The demographic data of patients and control are reported in Table 1. Specimens were harvested by Drs. Roberta Capucci and Alice Poggi, Obstetrics and Gynecology Clinic, University Hospital of Ferrara (Contini et al., 2018; Tagliapietra et al., 2019). Written informed consents were obtained from all females according to the Declaration of Helsinki. The province ethics committee of Ferrara, Italy, approved the study (ID: 151078).

DNAs/RNAs were exacted and purified from chorionic villi, as reported (Rotondo et al., 2015, 2017b). PBMCs were isolated from SA and VI blood samples with a density gradient centrifugation (Mazzoni et al., 2014, 2017b). DNA was then extracted from PBMCs using the QIAmp DNA Blood and Tissue Extraction Kit (Qiagen,

TABLE 1 Demographic data of SA patients and VI control

	SA	VI
Number of patients	100	100
Age of the patients (years, mean ± *SD*)	36 ± 5	32 ± 6
Number of pregnancies (mean ± *SD*)	2.4 ± 1.6	2.5 ± 1.2
Weeks of gestation (mean ± *SD*)	8.2 ± 1.3	10.2 ± 1.5

Abbreviations: SA, spontaneous abortion; VI, voluntary interruption.

Milan, Italy; Rotondo et al., 2017a). Tight precautions were taken to avoid cross-contamination during DNA extraction and analysis procedures. Indeed, specimens were purified simultaneously with a salmon sperm DNA (ssDNA) sample and distilled H_2O (Mazzoni et al., 2017a). After purification, DNAs/RNAs were quantified by spectrophotometric reading (Rotondo et al., 2018a; NanoDrop-2000, Thermo Fisher Scientific, Monza, Italy). Furthermore, DNA PCR suitability was evaluated amplifying the *β-globin* gene (Rotondo et al., 2017b). Nucleic acids were then stored at −80°C until the time of molecular analyses.

2.2 | ddPCR assay set up

ddPCR assay was set up in term of accuracy, sensitivity and specificity (Figure 1). The accuracy of the ddPCR assay was verified by testing scale dilutions of the pUC57MC1 recombinant plasmid containing MCPyV sequences (GenBank: EU375803) (Mazzoni et al., 2017a; Rotondo et al., 2017a). The linear range was assessed by scalar dilutions of the pUC57MC1 plasmid from 10^5 to 10^0 copies/μl. Experiments were performed three times. Experimental data and theoretical values were converted into logarithms and compared. The sensitivity, that is, lower detection limit, was determined testing 10^0 and 10^{-1} pUC57MC1 plasmid copies/μl. The specificity was assessed by testing two HPyVs, highly related to MCPyV, that is, JCPyV and BKPyV, as negative controls. Specifically, pMITCR1A and pBR322_BKPyV containing the complete genomes of JCPyV (GenBank: NC_001699.1) and BKPyV (GenBank: AB301099.1), respectively, were tested, together with pUC57MC1 plasmid (Rotondo et al., 2017a; Tagliapietra et al., 2019). Analyses were done including negative controls, that is, ssDNA and distilled H_2O (Tagliapietra et al., 2019).

2.3 | ddPCR assay for the detection and quantification of MCPyV DNA in clinical samples

MCPyV DNA load in SA and VI chorionic villi and PBMCs was evaluated using a specific ddPCR assay. This quantitative method, which allows to detect viral DNA sequences was performed using the QX200 Droplet Digital PCR System-Bio-Rad (Bio-Rad, Segrate, Milan, Italy; Mazzoni et al., 2017b; Tagliapietra et al., 2019). The ddPCR technique allows to analyze viral DNA load without a control because it provides an absolute detection of viral DNA sequences. The ddPCR reaction contains 11 μl of a 2 × ddPCR super mix (QX200 EvaGreen ddPCR, Bio-Rad, Segrate, Milan, Italy), 0.4 μl of each primer (final concentration ranging between 0.18 μM and 0.20 μM each) and 10.2 μl of DNA (~100 ng per reaction). Primers/probe used are as follows: RQ MCPyV_LT_1F 5′-CCACAGCCAGAGCTCTTCCT-3′, RQMCPyV_LT_1R 5′-TGGTGGTCTCCTCTCTGCTACTG-3′, RQMCPyV-Probe FAM-TCCTTCTCAGCGTCCCAGGCTTCA-TAMRA (Rotondo et al., 2017a). The mixture was added to the DG8 cartridge at the same time as 20 μl of droplet formation oil, using an automated droplet generator (Bio-Rad, Segrate, Milan, Italy). Every sample was divided into ~20,000 droplets. Then, generated droplets were transferred into a 96-well PCR plate, covered with pierceable foil, heat-sealed using a PX1 PCR Plate Sealer

(Bio-Rad, Segrate, Milan, Italy), and then placed in a thermal cycler (SimpliAmp, Applied Biosystem, Milan, Italy). The cycling conditions were as follows: 10 min at 95°C, 35 cycles of: 30 s at 94°C, 1 min at 57°C, and 10 min at 98°C. After PCR, the 96-well PCR plate was fixed on a plate frame and placed in the reader. Data were analyzed using the QuantaSoft analysis software (Bio-Rad, Segrate, Milan, Italy). A threshold line was employed to discriminate positive and negative droplets. Results were tested by the QuantaSoft analysis software (Bio-Rad, Segrate, Milan, Italy). The Poisson statistics was applied to calculate the absolute concentration of MCPyV DNA sequences in each sample. The cellular EIF2C1 gene was used to determine the human cell equivalents of each sample under analysis. Viral DNA load values were indicated as viral copies per 10^4 human cell equivalents (copy/10^4 cells).

2.4 | MCPyV gene expression analysis

MCPyV large T antigen (LT) and viral capsid protein 1 (VP1) mRNAs were retro-transcribed using the Improm II (Promega, WI) reverse transcription system, as reported elsewhere (Torreggiani et al., 2019). Then, complementary DNAs were analyzed for MCPyV LT and VP1 expressions (modified herein from Neumann et al., 2011) by qPCR using the SYBR chemistry. The primers employed were as follows: MCPyV_LT_F, 5′-CACACGGGACCAACTCAAG-3′; MCPyV_LT_R, 5′-AGGTATATCGGGTCCTCTG-3′; MCPyV_VP1_F 5′-AAAACACCCAAAAGGCAATG-3′, MCPyV_VP1_F, 5′-GCAGAGACACTCTTGCCACA-3′. The *GAPDH* gene was included in each reaction as the housekeeping gene for the expression analysis (Rotondo et al., 2015, 2018a). Data were analyzed employing the $2^{-\Delta\Delta Ct}$ method
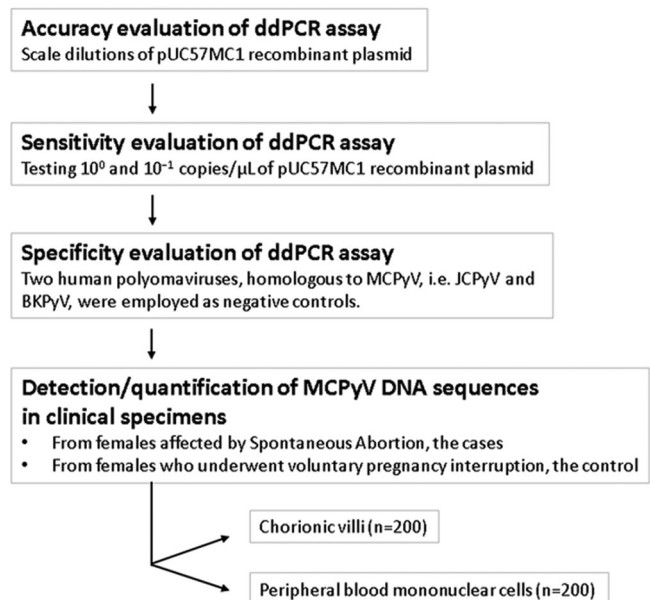
Flow chart of ddPCR assays

FIGURE 1 Flow diagram of ddPCR assays. ddPCR assays were set up to detect and quantify MCPyV DNA sequences in clinical specimens. ddPCR, droplet-digital polymerase chain reaction; MCPyV DNA, Merkel cell polyomavirus DNA

(Rotondo et al., 2018b). qPCR assays were performed three times, in triplicate/sample.

2.5 | Statistics

The prevalence of viral DNA sequences in SA and VI samples and viral DNA load values were statistically analyzed by the χ^2 test and the nonparametric Mann–Whitney-U test, respectively (Fainardi et al., 2016). Statistical analyses, including linear regression analysis of ddPCR accuracy, were carried out using Graph Pad Prism version 4.0 for Windows (Graph Pad, La Jolla, CA; Mazzoni et al., 2016, 2017b). p-values < .05 were considered statistically significant (Rotondo et al., 2018b).

3 | RESULTS

3.1 | Accuracy, sensitivity, and specificity of the ddPCR assay

The accuracy of the ddPCR assay was set up detecting serially 10-fold (from 10^5, 10^4, 10^3, 10^2, 10^1 to 10^0 copy/μl) diluted pUC57MC1 plasmid, which contains MCPyV DNA sequences (Figure 2a). Each dilution was analyzed three times, without difference. Results and calculated values were converted into logarithms and compared. Results indicate that the ddPCR method for the detection of MCPyV DNA sequences showed high accuracy, with an R^2 of 0.9938.

The sensitivity of ddPCR assay was determined employing 10^0 and 10^{-1} pUC57MC1 plasmid copies/μl of reaction volume. Recombinant plasmid DNAs (10^{-1} copy/μl), were detected only in one experiment, whereas all experiments revealed these controls at a concentration of 10^0 copy/μl. In our experimental conditions, the lower detection limit of the ddPCR method was 10^0 copy/μl.

To estimate the specificity of ddPCR assay, recombinant plasmids pMITCR1A and pBR322_BKPyV, containing the complete genomes of two HPyVs homologous to MCPyV, that is, JCPyV and BKPyV, were tested in ddPCR together with the pUC57MC1 plasmid. Results indicate that only pUC57MC1 plasmid tested positive, whereas other plasmids and controls were all negative, as expected (Figure 2b).

3.2 | MCPyV DNA sequences in chorionic villi and PBMCs

The specific ddPCR assay for the detection and quantification of MCPyV DNA sequences was employed to analyze clinical specimens, including chorionic villi and PBMCs from SA and VI females.

DNA sequences belonging to MCPyV were detected in chorionic villi (4/100, 4%; 5/100, 5%) from SA and VI, respectively. MCPyV DNA was revealed in PBMCs (9/100, 9%; 14/100, 14%) from SA and VI, respectively (Table 2; p > .05). Furthermore, three females from the VI group tested MCPyV-positive in both chorionic villi and PBMCs.

MCPyV DNA load in cell DNA from chorionic villi and PBMCs was determined using the same ddPCR method (Figure 3). The mean MCPyV DNA load detected in chorionic villi was 1.99 copy/10^4 cells (range 1.25–3.35 copy/10^4 cells) in the SA group (n = 4) and 3.02 copy/10^4 cells (range 1.42–6.05 copy/10^4 cells) in the VI group (n = 5; Figure 3a). Different MCPyV DNA loads revealed in SA and VI specimens were not statistically significant (p > .05). The mean MCPyV DNA load detected in PBMCs was 2.09 copy/10^4 cells (range 1.15–4.66 copy/10^4 cells) in SA (n = 9) and 4.09 copy/10^4 cells (range 1.5–17.55 copy/10^4 cells) in VI samples (n = 14; Figure 3b).
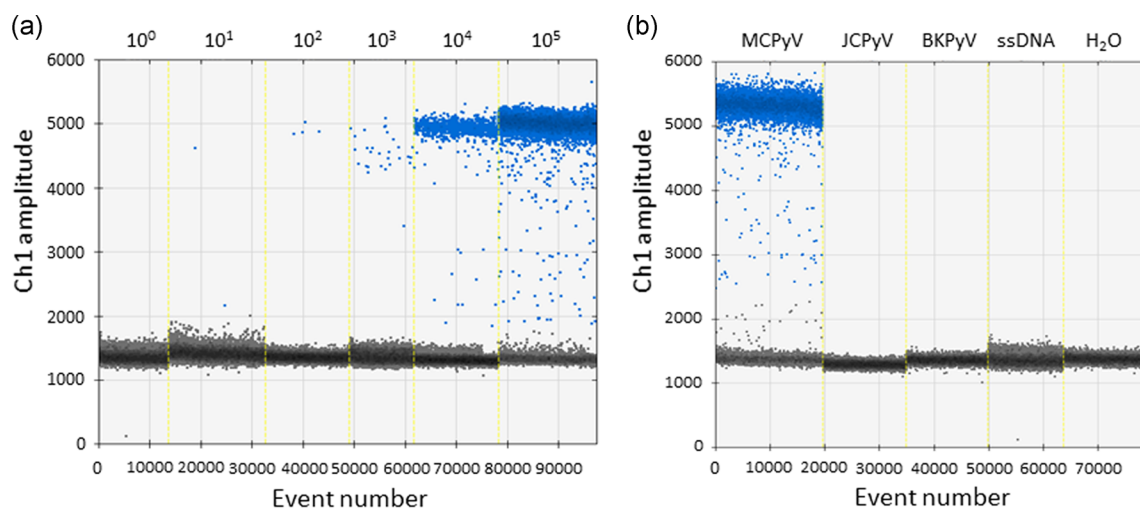

FIGURE 2 Sensitivity and Specificity of ddPCR. (a) Representative amplitude of the ddPCR amplified pUC57MC1 recombinant plasmid. The pUC57MC1 plasmid, carrying MCPyV DNA sequences, was serially diluted from 10^5 to 10^0 copies/μl (vertical lines). (b) Representative analysis of specificity of ddPCR. Vertical lines represent the fluorescent amplitude of pUC57MC1, pMITCR1A, and pBR322_BKPyV plasmids containing MCPyV DNA sequences (GenBank accession number EU375803), and complete genomes of JCPyV (GenBank accession number NC_001699.1) and BKPyV (GenBank accession number AB301099.1), respectively, and negative controls, including one control containing salmon sperm DNA (ssDNA) and an additional technical negative control (distilled H_2O). ddPCR, droplet-digital polymerase chain reaction; MCPyV DNA, Merkel cell polyomavirus DNA [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Prevalence of MCPyV DNA sequences in chorionic villi and PBMC samples

| Groups | Number of positive samples/samples analyzed (%) | |
	Chorionic villi	PBMCs
SA	4/100 (4)	9/100 (9)
VI	5/100 (5)	14/100 (14)
p value	.7561	.3757

Abbreviations: MCPyV DNA, Merkel cell polyomavirus DNA; PBMCs, peripheral blood mononuclear cells; SA, spontaneous abortion; VI, voluntary interruption.

Differences in DNA loads between SA and VI groups were not statistically significant (p > .05).

3.3 | MCPyV LT and VP1 gene expressions

MCPyV LT and VP1 transcripts were investigated in MCPyV-positive samples, that is, SA (n = 4) and VI (n = 5) chorionic villi. The expression of both MCPyV LT and VP1 mRNAs was detected in SA (2/4; 50%) and VI (2/5; 40%) specimens (p > .05). Furthermore, an additional SA sample (1/4; 25%,) tested MCPyV-positive for the LT mRNA, whereas VP1 mRNA was not detected. Quantitative analysis showed that MCPyV LT and VP1 were overexpressed by 3- and 3.9-fold, respectively, in SA compared with VI samples. However, no statistical differences in MCPyV LT and VP1 mRNA expressions were observed between SA and VI cohorts (p > .05).

4 | DISCUSSION

In the present study, an accurate, sensitive and specific method based on ddPCR system was set up for the detection and quantification of MCPyV DNA in SA.
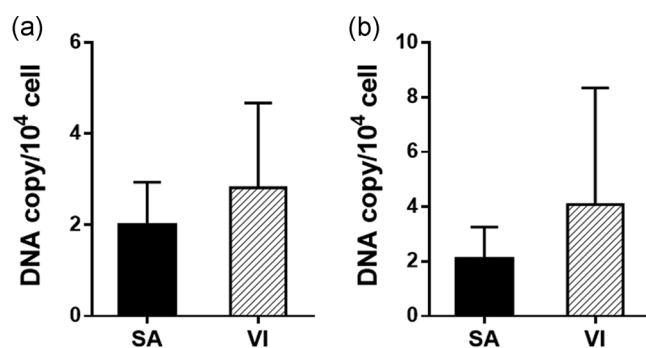

FIGURE 3 Mean viral DNA load detected by ddPCR. (a) Mean MCPyV DNA load (viral DNA copy/10^4 cells) in chorionic villi specimens from SA (n = 4) and VI (n = 5); (b) Mean MCPyV DNA load (viral DNA copy/10^4 cells) in PBMCs from SA (n = 4) and VI (n = 5). All panels: error bars represent standard mean deviation. The difference in viral load between the SA and VI groups was not statistically significant (p > .05). ddPCR, droplet-digital polymerase chain reaction; MCPyV DNA, Merkel cell polyomavirus DNA; PBMCs, peripheral blood mononuclear cells; SA, spontaneous abortion; VI, voluntary interruption

ddPCR assay accuracy was determined detecting scale dilutions of a MCPyV DNA recombinant plasmid and then comparing experimental data with the theoretical values. Calculations show a high goodness-of-fit between observed and theoretical values, thus indicating a high accuracy. These data indicate that the developed ddPCR method for the detection of MCPyV DNA is capable to quantify copies of viral DNA with high precision (Yang et al., 2017). Then, the sensitivity of the ddPCR assay was evaluated indicating as the lower detection limit, one copy/μl. This result indicates that the our ddPCR assay is an effective method for the quantification of MCPyV DNA, especially in detecting low concentrations of templates. Indeed, the solution subdivided in thousands of droplets increases the overall precision of the quantification (Pinheiro et al., 2012). The specificity of the assay was ensured by testing different plasmids containing the complete genomes of JCPyV and BKPyV, which are two HPyVs homologous to MCPyV (Rotondo et al., 2017a, 2017b; Tagliapietra et al., 2019). No cross-reactivity was observed, since none of the plasmids tested positive. These results indicate that the ddPCR assay presented a high specificity in detecting MCPyV DNA without the risk of cross-reactivity with other viral DNAs. Taken together, experimental data suggest that the our ddPCR method for the detection and quantification of MCPyV DNA is accurate and provides a high sensitivity and specificity. It has been largely demonstrated that ddPCR is capable in detecting few copies of target DNA with high precision and accuracy (Hindson et al., 2011; Pinheiro et al., 2012; Yang et al., 2017). On this ground, ddPCR has been employed to investigate a wide range of viral infections (Hall Sedlak & Jerome, 2014; Tagliapietra et al., 2019), including HPyV sequences (Mazzoni et al., 2017b; Tagliapietra et al., 2019).

In the present study, the capability of the ddPCR method to provide an analytical detection/quantification of MCPyV DNA in clinical samples was assessed. The lack of accurate investigations behind the role of MCPyV in SA prompted us to develop this new ddPCR assay. Indeed, previous studies indicated viral infections as risk factors for SA (Giakoumelou et al., 2016; Tagliapietra et al., 2019). ddPCR data on clinical samples analyzed herein indicate that MCPyV DNA is present in chorionic villi from SA and VI females with a low prevalence, without a significant difference. In addition, low MCPyV DNA loads were measured in both SA and VI cohorts. It is plausible that this result is related to the limited number of MCPyV-positive samples analyzed herein. Notably, a low-MCPyV DNA load has been assessed to have clinical relevance in the etiology of several nonMCC cancers, including brain cancers (Sadeghi et al., 2015), non–small-cell lung carcinoma (Behdarvand et al., 2017) and head and neck squamous cell carcinoma (Mohebbi et al., 2018), thereby suggesting that only a fraction of cancerous cells might be infected with MCPyV. Accordingly, our quantitative results indicate that only a subset of chorionic villi cells from both SA and VI females is infected by MCPyV. We may speculate that the low prevalence and the low viral DNA load revealed in SA and VI indicate that MCPyV is present in a latent/persistent infection (Krump, Liu, & You, 2018), without impacting the embryo development. This interpretation is further supported by viral gene expression data obtained in

MCPyV-positive chorionic villi. Indeed, MCPyV LT and VP1 mRNAs tested positive in both SA and VI specimens, without a significant difference between the two groups. Our data, together with the previously reported results (Sadeghi et al., 2010), indicate that MCPyV is not associated to SA.

Interestingly, MCPyV DNA was detected by ddPCR with a low viral DNA load in PBMCs from both SA (9%) and VI (14%). These data are in agreement with earlier results where a low viral DNA load was detected in buffy coats (22%; Pancaldi et al., 2011) and sera (2.4%; Mazzoni et al., 2017b) from healthy subjects.

Altogether our results support the feasibility/applicability of the ddPCR technique for quantitative and qualitative analyses of MCPyV DNA in clinical samples. In addition, the quantification of MCPyV DNA was achieved without the need of calibration curves (Beck et al., 2013; Sadeghi et al., 2010). This aspect makes this technique ideal for measurements of HPyVs DNA (Mazzoni et al., 2017b; Tagliapietra et al., 2019). Indeed, plasmids carrying HPyVs sequences, routinely used as positive controls in qPCR/PCR assays, are prone to circulate in laboratories, thereby increasing the risk to false-positive results (Shah, 2006).

In conclusion, in the present study, we set up and developed a specific and sensitive ddPCR method for the detection of MCPyV DNA in SA. The ddPCR assay displayed a high accuracy, sensitivity, and specificity. MCPyV DNA was identified with a low prevalence in chorionic villi from SA and VI. The few copies of MCPyV DNA detected in both chorionic villi and PBMCs may suggest that this virus might be present in a latent/persistent phase. Indeed, most of SA and VI MCPyV-positive samples expressed LT and VP1 mRNAs.

The new ddPCR method could provide a powerful tool to investigate the MCPyV infection in humans and its association with specific pathologies.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Study design/supervision: F. V., C. C., F. M., and M. T. Sample analysis/ validation, investigation, interpretation of data, and experiments execution: A. T., J. C. R., and L. O. G. Data curation and statistical analysis: J. C. R., E. M., I. B., L. O. G., and F. M. Writing, original draft preparation, and data visualization: J. C. R. and A. T. Critical revision/ discussion of the manuscript, formal analysis, and writing, review/ editing: F. V., C. C., F. M., and M. T. Administrative/technical/material support: I. B. Resources acquisition: I. B. Funding acquisition and project administration: C. C., F. V., M. T., J. C. R., and F. M.

FUNDING INFORMATION

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Mauro Tognon (iD) http://orcid.org/0000-0001-8269-7937
Fernanda Martini (iD) http://orcid.org/0000-0001-9137-0805

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