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Utility of droplet digital PCR for the quantitative detection of polyomavirus JC in clinical samples

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ABSTRACT

Background: Quantitative PCR (qPCR) is the standard molecular method for detection of polyomavirus JC (JCPyV) DNA reactivation in serum and cerebrospinal fluid (CSF) in patients at risk of progressive multifocal leukoencephalopathy (PML). Recently, digital PCR has shown potential benefits over qPCR in viral diagnostics.
Objective: To evaluate the performance of droplet digital PCR (ddPCR) assay in assessing JCPyV-DNA status in clinical samples of patients at risk for PML.
Study design: JCPyV specific ddPCR was developed with primers/probes targeting Large T and the noncoding control region used in qPCR. The ddPCR accuracy of JCPyV-DNA quantification was investigated using serial dilutions of genomic JCPyV-DNA. The ddPCR JCPyV-DNA quantification and qPCR confirmation were performed on 150 CSF and 100 serum clinical samples.
Results: Using genomic JCPyV-DNA, ddPCR was highly sensitive, repeatable and reproducible for both molecular targets. Using clinical samples, JCPyV-DNA was detected in 13% of CSF and in 50% of serum samples with limit of detection of 30 copies/ml. Among the 19 JCPyV-DNA-positive CSF detected using the ddPCR, 15 also tested positive with the qPCR. Among the 50 JCPyV-DNA-positive serum identified with ddPCR, 41 tested positive with qPCR. All the ddPCR-negative samples were negative when assessed using qPCR. Additionally, the mean JCPyV-DNA viral load obtained with ddPCR in all samples was not significantly different from that of qPCR.
Conclusion: The results demonstrate that ddPCR is a highly sensitive alternative for measuring JCPyV-DNA that should be considered in clinical diagnostic testing of JCPyV-DNA in patients at risk of PML and other associated diseases.

1. Background

Human polyomavirus JC (JCPyV) is a virus that persists asymptomatically in the human population since childhood, and it has been associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system [1]. PML, a rare disease that is caused by reactivation of JCPyV replication in oligodendrocytes in patients with lymphoproliferative disorder, is more frequently diagnosed in human immunodeficiency virus infected patients [1]. Recent innovative therapies, such as those using novel monoclonal antibodies, can increase the risk of JCPyV reactivation and contribute to the development of PML [2–4]. In this context, rearrangements in the noncoding control region (NCCR) of JCPyV have been identified and are thought to be involved in the development of PML [1]. Additionally, JCPyV may lead to other neurological disorders such as granule cell neuronopathy and encephalopathy and may also cause meningitis [5].

In clinical practice, monitoring for JCPyV-DNA in the serum and cerebrospinal fluid (CSF) is required in order to assess the risk of PML [2]. However, because JCPyV can be detected in the blood of healthy people, and some PML patients are not viremic, the significance of JCPyV viral load in the blood remains controversial [3–7].

Abbreviations: JCPyV, Polyomavirus JC; NCCR, noncoding control region; DDPCR, droplet digital PCR; qPCR, quantitative real-time PCR; NTC, no template control; CSF, celebrospinal fluid; PML, progressive multifocal leukoencephalopathy.

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Moreover, although a CSF analysis is the main tool for assessing risk of PML, along with clinical and neuroradiological evidence [8], the JCPyV viral load in CSF may be low and needs a sensitive standard molecular diagnostic method for detection. Currently, quantitative real-time PCR (qPCR) is the standard molecular diagnostic method for monitoring JCPyV-DNA. More recently, a Multiplex qPCR assay targeting Large T and NCCR region was developed. The Multiplex qPCR assay is capable of detecting JCPyV-DNA with simultaneous discrimination of non-pathogenic and pathogenic variants [9]. The main benefit of the multiplex qPCR assay is that it can detect the presence of potentially virulent variants hiding within a patient thus indicating a greater risk for possible development of PML. However, qPCR has some limitations in accuracy, standardization, and precision. Indeed, qPCR depends on the relationship of the cycle threshold and the calibration curve of a standard DNA sample. In this context, marked variations in assay performance characteristics and differences in standard curve calibration, even when using commercial kits and standardized protocols, as well as the assay system that is utilized can prevent reproducible results [10].

Recently, digital PCR has shown potential to be more efficient in viral diagnostics with benefits over qPCR, such as absolute quantification without dependence on external standard curves [10,11]. The droplet digital PCR (ddPCR) generates droplets of PCR volume (theoretically at least 20,000 droplets), performs a PCR microreaction in each droplet, and uses the number of positive reactions, together with Poisson's distribution, to produce a direct, high confidence measurement of the original concentration [12,13].

2. Objective

To evaluate the performance of the ddPCR assay and to quantify and identify JCPyV-DNA in samples obtained from patients at risk for PML.

3. Study design

3.1. Samples

The present study involved an anonymous retrospective analysis of 150 CSF samples, obtained from patients with suspected PML, and 100 serum samples, obtained from HIV infected patients attending the Infectious Disease unit of the Careggi University Hospital, University of Florence. All samples were collected at the Clinical Microbiology and Virology unit, Careggi University Hospital, University of Florence.

3.2. JCPyV-DNA ddPCR absolute quantification

JCPyV infection was assessed extracting DNA from 200 μl of serum and CSF samples using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. Extracted DNA from serum and CSF samples or DNA from plasmid containing JCPyV-DNA genome was amplified with a ddPCR absolute quantification assay by using primer and probes targeting Large T antigen (JCT-3F forward primer 5′-AGTGTTGGGATCCTGTGTTTTCA-3′, JCT-4R reverse primer 5′-GTGGGATGAAGACCTGTTTTGC-3′ and TaqMan MGB JCT-1.2 probe labeled with FAM FAM-5′ CATCACTGGCAAACAT 3′) and NCCR (JRR-1F forward primer 5′-GGAGCCCTGGCTGCAT-3′, JRR-2R reverse primer 5′-TGTGATTAAGGACTATGGGAGG-3′ and TaqMan MGB JRR-1.1 probe labeled with VIC VIC-5′ CTGGCAGTTATAGTGAAACC-3′) specifically designed to identify virulent (PML-prototype) and non-virulent (Archetype) JCPyV variant [9]. The ddPCR reaction mixture consisted of 10 μl of a 2X ddPCR super mix (Bio-Rad), 0.5 μl of each JCPyV primer/probe mix (final concentration of 0.25 μM for each primer and probe), 5 μl of sample nucleic acid solution and RNA-free H2O in a final volume of 20 μl. The entire reaction mixture was loaded into a disposable plastic cartridge (Bio-Rad) together with 70 μl of droplet generation oil (Bio-Rad) and placed in the droplet generator (Bio-Rad). After processing, the droplets generated from each sample were transferred to a 96-well PCR plate (Eppendorf). PCR amplification was carried out on a T1000 thermal cycler (Bio-Rad) using a thermal profile of beginning at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 60 °C for 60 s, 1 cycle of 98 °C for 10 min, and ending at 4 °C. After amplification, the plate was loaded on the droplet reader (Bio-Rad), and the droplets from each well of the plate were read automatically. ddPCR data were analyzed using QuantaSoft analysis software (Bio-Rad), and the quantification of the target molecule was presented as the number of copies per 20 μl of reaction.

3.3. ddPCR spiking experiments

JCPyV-DNA and BKPyV-DNA control plasmid diluted in water were spiked into DNA solution extracted from CSF and serum obtained from JCPyV-DNA negative subject. Both plasmids were spiked into each of the extracted samples, with the concentration of BKPyV plasmid remaining constant (10^3 copies per 20 μl of reaction), while JCPyV covered 10-fold dilutions from 10^1 to 10^5 copies per 20 μl of reaction.

3.4. JCPyV-DNA real-time PCR quantification

The same DNA extracted samples that were amplified with ddPCR, were used in qPCR without any additional freezing-thawing process. Briefly, each DNA extracted sample was amplified with qPCR assay by using the same primers/probes targeting Large T and NCCR described for ddPCR. The lower limit of detection of the assay was 10 copies per milliliter of CSF and serum samples [9,14].

4. Results

4.1. JCPyV ddPCR accuracy

Firstly, the dynamic range of ddPCR for JCPyV-DNA was calculated using ten-fold serial dilutions (plasmid nominal copies number of 10^0–10^5 copies per 20 μl of reaction) of a plasmid containing the whole JCPyV-DNA genome carrying the NCCR archetype structure. Fig. 1 (panels A and B) shows an example of the readout ddPCR generated using QuantaSoft software, proving the ability of ddPCR to produce positive droplets down to the 10^0 dilution for the JCPyV plasmid tested.

In order to assess the sensitivity of the ddPCR, we repeated the assay using two-fold serial dilutions (plasmid nominal copies number of 100-1.6 copies per 20 μl of reaction) of the input JCPyV-DNA plasmid. As reported in Fig. 1 (panels C and D), we confirmed that ddPCR was highly sensitive under these conditions producing ddPCR positive droplets down to 3 copies of JCPyV-DNA per reaction. Moreover, the ddPCR values that were obtained were highly correlated with the input nominal copies number used showing marked linearity ($R2 > 0.98$, Fig. 2).

The reproducibility of the ddPCR assay was determined by measuring ten-fold serial dilutions (plasmid nominal copies number from 10^1 to 10^5 copies per 20 μl of reaction) in triplicate within the same experiment (to assess intra-assay variation) and in different experiments carried out on 3 different days (to assess inter-assay variation). The intra-assay coefficient of variation ranged from 0.01 to 2.17 and from 0.01 to 1.60 for Large T and NCCR target amplification, respectively (Table 1). The inter-assay coefficient of variation obtained ranged from 0.05 to 5.55 and from 0.05 to 2.22 for Large T and NCCR target amplification, respectively (Table 1).