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Multiplex qPCR assay for ultra sensitive detection of JCV DNA with simultaneous identification of genotypes that discriminates non-virulent from virulent variants

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Abstract

Background—JC Virus (JCV) is the etiologic agent for progressive multifocal leukoencephalopathy (PML), a demyelinating disease occurring in the brain of patients with underlying immune compromised states. All viable JCV genomes contain a conserved region in the T protein coding nucleotide sequence that when detected by PCR in CSF is a confirmatory diagnostic marker for PML along with clinical and neuroradiological evidence. The non-coding regulatory region (NCRR) is hypervariable, as evidenced by nucleotide sequence of the non-virulent variant, which is predominantly excreted in urine, versus that of virulent variants found in brain and CSF of PML patients. All variants can be found in blood.

Objective—A single assay that quantifies and identifies JCV DNA in clinical samples and discriminates between variants has significant value to physicians and patients at risk for PML.

Study Design—Separate primer pairs were tested together to quantitatively detect conserved viral DNA nucleotide sequence in patient samples, while simultaneously detecting the NCRR specific for the non-virulent variant.

Results—In testing using control plasmids and patients' CSF, blood, and urine, PML patients predictably demonstrated the non-virulent, archetype NCRR in urine, but virulent NCRR variants in CSF and blood.

Conclusion—The JCV qPCR Multiplex assay targets two regions in JCV genomes to simultaneously identify and measure viral DNA, as well as distinguish between variants associated with PML and those that are not. The multiplex results could signal risk for PML if patients are viremic with JCV variants closely associated with PML pathogenesis.

1. Background

The human polyomavirus, JCV, is the etiologic agent of the CNS demyelinating disease progressive multifocal leukoencephalopathy (PML), a relatively rare disease affecting immune compromised patients. The incidence of PML is highest in HIV-1 infected individuals approximating 3/100 and is an AIDS defining illness (1). In MS patients who

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have received greater than 24 doses of the monoclonal antibody therapy directed to α 4 integrins, natalizumab, and a history of immune suppressive therapy, the incidence of PML is 1/80 (2).

There have been more than 330 cases of natalizumab associated PML in MS patients. We have provided the laboratory confirmation in nearly half of those cases. There is still no effective treatment for PML, nor an animal model to investigate pathogenic mechanisms. However, JCV infection is globally ubiquitous with more than half the population having been exposed, the percent increasing with age. Approximately 30% of exposed individuals will become latently infected in kidney uroepithelial cells, excreting high viral copy numbers in urine without evidence of pathologic consequence in any tissue, including the brain (3). The genotype of urine excreted virus considered non-pathogenic, termed 'archetype,' has a unique 267 base pair arrangement of non-coding regulatory region nucleotide sequence (NCRR) and has rarely been associated with PML. However, select deletions and duplications in the archetype NCRR that result in direct tandem repeats may give rise to the arrangement of pathogenic variants found (4, 5). It is unclear in what tissues, or cells, such alterations could occur. The most likely candidate sites are lymphoid tissues, including bone marrow where JCV can persist latently (6, 7). Potentially pathogenic, non-archetype variants have deletion of nucleotide sequence in the 'd' sequence section of the archetype NCRR (4). Despite the hypervariability of the NCRR, all JCV genotypes have similar T protein coding sequences. Because the T protein coding sequence identified by the JCT primers/probe set of our CLIA JCV qPCR protocol (12) is necessary for viral growth, alterations in this conserved region result in non-viable virus (10, 11). Also, this coding sequence, located just after the splice site for small t, is unique. Therefore, DNA amplification in this region is specific for all JCV variants, but not other human polyomaviruses. Consequently, qPCR of the conserved JCT region provides a measure of JCV copy number regardless of variant origin (12). Amplification of viral DNA from CSF samples using this primers/probe set has proved highly sensitive and specific, and is the basis for the laboratory confirmatory diagnostic marker for PML. However, qPCR with these T primers and probe alone cannot distinguish the archetype variant from any potentially pathogenic variants. Direct nucleotide sequencing of the viral DNA recovered from the CSF is necessary to make that distinction, but difficult from low copy number samples, time consuming and costly. JCV DNA is also detected in the blood in non-PML patients, which is not surprising for a widely spread DNA virus. Although viremia is not by itself a diagnostic aid for PML, it can be a considerable factor in patients with underlying immune compromised conditions (13). Consequently, identifying JCV genotypic variants as non-virulent, or virulent, provides value in determining which patients may be at greater risk of developing PML. A previous qPCR assay described as multiplex distinguishes JCV from other DNA viruses (14), but does not distinguish JCV variants as detailed in the following.

2. Objective

A single assay that quantifies JCV DNA in clinical samples and discriminates between virus variants has significant value in assessing risk for PML.

3. Study Design

3.1 Primers and Probes

Table 1 shows the NCRR nucleotide sequence arrangement of two major variants: archetype, represented by the urine isolate CY (8); and tandem-repeat/prototype, derived from the original isolate of JCV Mad-1 (9) from PML brain tissue. The location of multiplex primers and probes for T protein sequence (JCT), and the NCRR of the archetype variant (JRR), are identified in relation to both of these approximately 5.1kb genomes. JCT

multiplex primers (JCT-3 and JCT-4) are in the same location of the N terminus of the viral T protein coding region as primers used in our CLIA JCV qPCR protocol (12), except the multiplex primers were designed two nucleotides shorter. The 5' end of the multiplex T protein internal probe (JCT-1.2) is labeled with a fluorescent reporter dye, 6-carboxyfluorescein (FAM), and has a minor groove binding (MGB), nonfluorescent quencher (NFQ) on the 3' end. As a set, the multiplex JRR primers (JRR-1 & JRR-2) and internal probe (JRR-1.1) are consistent with the known archetype NCRR sequence (8), spanning the 'd' sequence section minus 4 base pairs. Shown in red, JRR-1.1 is labeled with a fluorescent reporter dye (VIC) on the 5' end, and an MGB, NFQ at the 3' end. All primers and probes were synthesized to specification by Applied Biosystems of Life Technologies, Carlsbad, CA. The numbered nucleotide location and sequence of each primer and probe are given. The reaction parameters for the multiplex are identical to those for our previously published CLIA JCV qPCR protocol (12).

3.2 Positive Control and Standard plasmids

As a representative virulent variant control sequence, the JCV prototype (MAD-1) plasmid, pM1TC (9), was used. For the non-virulent variant control sequence, the JCV archetype plasmid, pCY (8), was used. To construct a standard curve DNA concentrations for each of these plasmids were established over a range from 100 pg to 10 attograms (ag) in eight decreasing 10 fold serial dilutions.

3.3 Multiplex Dual Spiking Experiments for Specificity

DNA spiking experiments were conducted to test whether different concentrations of the two control plasmids in the same PCR reaction influenced the amplification efficiency of either variant. Control plasmid standards diluted in water were spiked into DNA extraction solution from CSF, PBMC, plasma, and urine. Both control plasmids were spiked into each of the extraction samples, with the concentration of one plasmid remaining constant (10 fg), while the other covered the eight, 10 fold dilutions from 100 pg to 10 ag. The same procedure was also performed, but with the concentration layouts of the control primers exchanged. The CSF, PBMC, plasma, and urine were obtained from negative healthy donors. No significant changes were observed in amplification efficiency of either control plasmid due to competing plasmid concentrations, or presence of DNA extracted from the CSF, PBMC, or urine (data not shown). DNA extraction solution from plasma, however, showed some reduced amplification efficiency at 10 ul, but none at 5 ul. Therefore, in testing clinical plasma samples, both 10 and 5 ul volumes were run to normalize for this potential inhibition.

3. Results

The data in Figure 1 show amplification of a 'standard' plasmid (pCY) containing archetype JCV DNA (A, B), or pCY together with another standard plasmid (pM1TC) containing prototype JCV DNA (C, D). These amplifications were performed using the multiplex qPCR assay with the primers/probe set for JCV large T (JCT) and the 'd' sequence section of archetype NCRR (JRR). Because the archetype DNA contains both target DNA sequences, ten fold serial dilutions of pCY demonstrated similar Ct values (A) and linearity (B) for amplification of both JCV large T (black lines) and the archetype NCRR 'd' sequence section (red lines). Sensitivity of the multiplex assay was similar to what we achieved with the CLIA JCV qPCR protocol at 10 copies/ml (12). Ten fold serial dilutions of pM1TC, each spiked with only 10 fg of pCY showed (C) detection of only the large T from the prototype variant, until a Ct value of approximately 26 when both prototype and spiked archetype DNA were identified. Conversely, ten fold serial dilutions of pCY, each spiked with only 10 fg of pM1TC showed a multiplex amplification plot (D) similar to that seen

versus only the archetype (as in A). At higher Ct values, the presence of the prototype variant was evident in large T detection (black lines) that did not proceed below the concentration of the spike.

Multiplex Ct values of a clinical sample in which large T sequences are greater than detectable archetype sequence demonstrate the presence of additional genotypic variants. Interpretation of amplification from a clinical sample in which no archetype is detected would predict the presence of a virulent variant, as in CSF of a PML patient. In laboratory derived 'archetype only' samples, both large T and archetype would have similar Ct values demonstrating that only archetype, the non-virulent variant, is present.

Data in Fig. 2 show the clinical utility of the multiplex qPCR assay in two PML patients with different underlying diseases and risk: 1. amplification of clinical samples from a PML patient with AIDS shows CSF (A) with only large T detection indicating presence of a virulent variant, plasma (B) taken close to time of PML diagnosis indicating the presence of only a virulent variant, and urine (C) taken close to time of PML diagnosis with high copies of archetype; 2. an identical profile was seen in samples from a natalizumab treated MS/PML patient in the CSF (D), plasma (E), and urine (F). The multiplex assay has been used in testing several hundred CSF samples, as well as urine, plasma and serum, as indicated in Table 2. The results confirm the presence of virulent forms of JCV DNA in CSF of PML patients, and predominantly the non-virulent (archetype) in urine.

4. Discussion

There have been more than 330 cases of PML in MS patients under treatment with the α integrin blocker natalizumab. We have provided the laboratory confirmation in nearly half of those cases. Current qPCR assays, including the CLIA validated/certified assay in this laboratory (CLIA Registration 21D0959140, CMS, HHS), target the conserved T protein coding sequences that identify the presence of JCV DNA regardless of nucleotide variations located in other regions of JCV viral genomes. This assay is very sensitive and specific in this laboratory, allowing detection down to 10 copies/ml from CSF, plasma, serum, or urine using the Qiagen DNA spin column kit for template extraction (15). Other qPCR assays have evaluated variations of the human polyomavirus BKV sequence using multiple sets of primers and probes over the same genomic region without significant differences in assay results (16, 17). The assay we have describe in this manuscript, however, adds a new dimension to the CLIA certified JCV qPCR in the form of a multiplex that targets an additional sequence unique to the archetype non-coding regulatory region (NCRR). The archetype variant is rarely associated with pathological consequences of JCV infection and not considered a variant that causes PML (18, 19). While there can be alterations within the canonical archetype 66 base pair insert (otherwise known as the 'd' sequence section), the presence of these variants in PML patient CNS samples is extremely rare and only detected long after PML diagnosis has been made. Also, as archetype is defined in this assay (8), the lack of archetype sequences in clinical samples is a key feature of the multiplex assay. Therefore its identification in plasma/serum, PBMCs, or other tissues does not carry the same risk for PML as would, for example, the prototype variant. The multiplex assay then discriminates between the non-virulent and virulent variants by either detecting the archetype alone or by detecting JCV DNA that does not have the archetype genotype. For example, if a plasma sample has detectable copies of viral DNA measured by amplification of the JCV T region, but no detectable archetype DNA, then the sample contains potentially virulent variants. If the plasma sample was taken from a non-PML patient with an underlying disease or therapy that has a risk for PML, then the presence of potentially virulent variants in the circulation could be considered an additional risk for PML. The JCV DNA multiplex assay provides that information from qPCR analysis of a single template, in

a one assay format, using large T and archetype primers/probe sets. An important utility of this multiplex assay would be testing urine and/or plasma samples over time from patients in risk categories (i.e. immune-compromised conditions). Any increase in the detection of large T sequences over archetype would indicate the multiplication of potentially virulent variants, indicating a greater risk for possible development of PML.

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Figure 1.

Multiplex quantitative real-time PCR using the JC virus non-coding regulatory region primers/probe set JRR-1, JRR-2, JRR-1.1-VIC (red), in addition to the JCV large-T primers/probe set JCT-3, JCT-4, JCT-1.2-FAM (black), versus eight concentrations (100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 ag, 10 ag) of archetype JCV plasmid (pCY): **A** amplification plot showing the change in fluorescence (Delta Rn) in respect to PCR cycle number (Cycle#) with plasmid concentrations ranging downward from left to right; **B** standard-curve shown in the cycle number read at threshold (Ct) versus log of the plasmid copy number for both primers/probe sets (JRR, red line and shaded-diamond data points; JCT, black line and unshaded-square data points) with plasmid concentrations ranging upward from left to right. This amplification plot and standard-curve represent a set of quadruplicate reactions run together on a single plate, and are representative of numerous replicate assays.

Amplification plots utilizing primers/probe sets as above (for **A** and **B**), but versus **C** eight concentrations (100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 ag, 10 ag) of prototype JCV plasmid (pM1TC), each spiked with one concentration (10 fg) of archetype pCY; **D** eight concentrations (100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 ag, 10 ag) of archetype pCY, each spiked with one concentration (10 fg) of prototype pM1TC. Both amplification plots **C** and **D** show the change in fluorescence (Delta Rn) in respect to PCR cycle number (Cycle#) with plasmid concentrations ranging downward from left to right. Note that the 10 fg archetype pCY and prototype pM1TC spike in **C** and **D**, respectively, results in the lower concentration (10 fg, 1 fg, 100 ag, 10 ag) large T plots (black lines) crossing the threshold (t) closely together in the same general area. Both amplification plots **C** and **D** represent a set of quadruplicate reactions that were grouped and run together on a single plate. The thresholds (t) for all analyses are set at a consistent Delta Rn level and fall appropriately in the geometric phase of these amplifications.

Figure 2.

Amplification plots from multiplex quantitative real-time PCR (qPCR) using the JC virus non-coding regulatory region primers/probe set JRR-1, JRR-2, JRR-1.1-VIC (red), in addition to the JC virus large-T primers/probe set JCT-3, JCT-4, JCT-1.2-FAM (black), versus total DNA extracted from 200 μ l for each of three matched samples from PML patients: **A** CSF, **B** plasma, **C** urine of an HIV-1/AIDS patient; and **D** CSF, **E** plasma, **F** urine from a natalizumab treated MS patient. The thresholds (t) are set at a consistent Delta Rn level and fall appropriately in the geometric phase of these amplifications. These data show similarities (similar patterns) to data generated with the same assay on numerous matched samples from other PML patients. Also, in examining qPCR assay efficiencies, similar copies/ml (c/ml) were achieved between the multiplex and qPCR using only the large T primers/probe set (JCT) versus each sample type from the natalizumab treated MS patient. Comparative c/ml in terms of the multiplex qPCR versus the large T primers/probe

set qPCR (multiplex / JCT) were: CSF ($8.3^2 / 7.7^2$); plasma ($4.65^3 / 5.73^3$); and urine ($3.3^8 / 1.8^8$).

Schematic comparison between the prototype (Mad-1) and archetype (CY) JC virus (JCV) genomes, including the location of primers/probe sets used in multiplex quantitative real-time PCR (qPCR) analyses. JCV genomes are circular, supercoiled, double-stranded DNA with an early (E) protein coding region (large T and small t) transcribed in one direction from a single strand, and a separate late (L) protein coding region (agnoprotein, Vp1, Vp2 and Vp3) transcribed in the opposite direction from the complementary strand. Between start-sites for the early and late coding regions, and including the origin of DNA replication (ORI), is the non-coding regulatory region (NCRR). Dots on the depicted circular genome represent non-coding nucleotide sequences, including the NCRR, other intergenic regions, and the spliced portion of large T message. The NCRR contains a hypervariable region (*v*) which can be used to distinguish variant JCV genomes. JCV variants differ in levels of viral activity according to the unique nucleotide sequence of the *v* region. While the archetype JCV is considered non-pathogenic, the prototype JCV and most other non-archetype genomes range in pathogenic activity. Unbroken dual-circles depict the archetype JCV genome, while broken, outer dual-circles locate deleted and repeated nucleotide sequences in the prototype JCV. The highlighted linear NCRR comparison between Mad-1 and CY utilizes nucleotide-numbering systems adapted from the prototype JCV genome and the archetype JCV NCRR respectively. The archetype NCRR contains a single copy of all the nucleotide sequence sections observed in the NCRR of all other JCV variants. These nucleotide sequence sections are designated *a*, *b*, *c*, *d*, *e* and *f*. The unique arrangement, repetition, and/or deletion of the sequence sections in the *v* region identify a JCV variant. Variants that are not the archetype have deletion of nucleotide sequence in the *d* section (e.g., the *d* section is completely absent from the prototype JCV); therefore, qPCR amplification based on a primers/probe set (JRR-1, JRR-2, and JRR-1.1-VIC) that span the entire *d* sequence can be used to determine the presence of the archetype variant in patient samples.

Table 1

Specimens		JCV DNA detect total		PML Patients with JCV genomes			Non-PML Patients with JCV genomes			copies/mL of JCV genomes detected		
Type	Total	Specimens	Patients	Totals	P ^a	P/A ^b	A ^c	Totals	P ^a	P/A ^b	A ^c	Median
CSF	222	100	52	52	43	6	3	0	0	0	0	1.0e1
Plasma	83	41	35	8	7	1	0	27	17	9	1	1.2e1
Serum	36	7	4	2	1	1	0	2	1	0	1	1.3e1
Urine	20	12	6	0	0	2	2	2	1	0	2	1.5e1
												3.3e1
												4.6e3
												4.7e3
												9.7e1
												1.2e2
												3.0e6
												4.3e8
												1.5e5
												1.3e1
												2.7e4
												4.5e8
												3.9e8

^a potentially pathogenic only,
^b both potentially pathogenic and archetype,

Table 2

Data generated from use of the multiplex quantitative real-time PCR (qPCR) assay allowed for the determination/identification of JCV genomic variants [^a potentially pathogenic (P) including prototype, ^c the non-pathogenic archetype (A), and/or ^b both (P/A)] that were present in PML and non-PML patients from whom various clinical specimen were available (CSF, plasma, serum, and urine). While no JCV genomes were detected in the CSF of non-PML patients (as expected for a patient clinically defined as “non-PML”), potentially pathogenic (P) variants in the CSF of PML patients were predominant despite the archetype (P/A, plus A) also being present in some of these CSF specimens. Plasma and serum of PML and non-PML patients demonstrated both potentially pathogenic and the archetype (P, plus P/A), while only two non-PML patients demonstrated the archetype alone (A) in blood. Urine of PML patients demonstrated both potentially pathogenic and archetype (P/A, plus A), while only archetype (A) was detected in urine of non-PML patients. Ranges of JCV genome copy numbers detected by the multiplex qPCR are also given for each specimen type tested. Despite archetype being the predominant variant detected in the urines tested, detection of other variants excreted was also shown from these PML patients who have very high copy number of potentially pathogenic variants circulating in their blood and CSF.