Association of Neurotropic Viruses in HIV-Infected Individuals Who Died of Secondary Complications of Tuberculosis, Cryptococcosis, or Toxoplasmosis in South India

Rajesh Kannangai, Jaiprasath Sachithanandham, Anita Mahadevan, Asha Mary Abraham, Gopalan Sridharan, Anita Desai, Vasanthapuram Ravi, Susarla Krishna Shankar

Department of Clinical Virology, Christian Medical College, Vellore, India; Department of Neuropathology, NIMHANS, Bangalore, India; Sri Sakthiamma Biomedical Research Institute, SNHRC, Vellore, India; Department of Neurovirology, NIMHANS, Bangalore, India

The frequencies of 10 opportunistic DNA viruses were determined by multiplex real-time PCR in paired cerebrospinal fluid (CSF) and brain tissue of HIV-infected individuals. In the CSF, viruses were detectable in 45/55 cases: JC virus (JCV) in 62%, Epstein-Barr virus (EBV) in 44%, cytomegalovirus (CMV) in 25%, varicella-zoster virus (VZV) in 3.6%, herpes simplex virus 1 (HSV-1) in 1.8%, and human herpesvirus 6 (HHV-6) in 1.8% of cases. A single virus was detectable in 20 cases, 19 cases had coinfection with two viruses, and 6 cases were positive for three viruses. JCV was detectable in the CSF of 62% of cases and in 42% of brain tissues, with higher loads in progressive multifocal leukoencephalopathy (PML) (P < 0.05).

Several viruses can cause opportunistic infections in HIV-infected individuals, with high morbidity and mortality in HIV-infected individuals (1). These infections are more frequently reactivation of the latent viruses in the host. It is essential to identify the causative agent of these opportunistic infections to initiate therapy or prophylactic treatment. As the clinical pictures caused by different viruses often overlap and multiple infections are also frequent in immunocompromised hosts, simultaneous screening of a wide range of viruses would be cost-effective management (2). Viruses like Epstein-Barr virus (EBV), cytomegalovirus (CMV), and JC virus (JCV) can result in life-threatening infections in the central nervous system (CNS) of immunodeficient individuals (3, 4). The clinical conditions include primary CNS lymphoma, cytomegalovirus encephalitis, and progressive multifocal leukoencephalopathy (PML) (5–7). To compound the clinical problem, viral infections can coexist with bacterial and fungal infections. Currently, there are no reports from India on the frequency of CNS viral infections among HIV-1-infected individuals. The aim of this study was to develop a real-time multiplex PCR for the simultaneous detection of DNA viruses in paired CSF and brain tissue samples of HIV-infected individuals.

The study was carried out in the Departments of Clinical Virology (site 1) and Neuropathology (site 2) of two tertiary care centers in south India. The study was approved by the institutional review boards of both institutions. Brain tissues and CSF samples of histopathologically well-characterized HIV-associated opportunistic infections collected at autopsy and archived in the Human Brain Tissue Repository of Neuropathology were utilized for the study. All the samples archived were collected at autopsy following written informed consent from the legal close relative(s) of the deceased to use the biological material for research. A total of 55 archived paired CSF and brain tissue samples collected from HIV-positive individuals who had died with confirmed bacterial or fungal infections were investigated. These diagnoses were confirmed by histopathology, immunohistochemistry, and/or microbiological methods (culture, serology). The study also included 20 CSF samples collected from patients who died of noninfectious disease, like malignancy, or road traffic accidents. Brain tissue samples were available from 10 of these individuals. These samples were used as disease-free controls.

DNA was extracted from CSF samples using the commercially available QIAamp DNA blood minikit (Qiagen, Hilden, Germany) and from brain tissue samples using the QIAamp DNA minikit (Qiagen, Hilden, Germany). Sterile Milli-Q water was used as a negative control and was included after every 5th sample in each run of extraction to check the PCR cross-contamination. The multiplex real-time assay was done on PCR Rotor gene RG-3000/6000 (Corbett Research, Sydney Australia). The primers were used in four cocktail mixes as follows: cocktail 1, CMV (8), EBV (8), and herpes simplex virus 1 (HSV-1) (8); cocktail 2, varicella-zoster virus (VZV) (8) and human herpesvirus 6 (HHV-6) (8); cocktail 3, JCV (8) and HSV-2 (8); cocktail 4, HHV-8 (8), BK virus (BKV) (9), and adenovirus (10).

The samples found positive for respective viruses in initial screening multiplex PCR were tested by individual in-house quantitation assays. DNA integrity of all the PCR-negative samples was confirmed by testing for a 135-bp segment of the human endogenous retrovirus 3 (ERV-3) envelope gene (11), and the ERV-3 assay is also used to determine the copy numbers of viruses in tissue. The lower detection limits for all the viruses used in the study were as follows: HSV-1, 0.0056 50% tissue culture infective doses (TCID50)/10 μl input (0.56 TCID50/ml); HSV-2, 0.0031 TCID50/10 μl input (0.31 TCID50/ml); VZV, 0.001 PFU/10 μl input (0.1 PFU/ml); EBV, ≤1 plasmids/10 μl input (<100 copies/ml); CMV, 1 to 10 copies/10 μl input (100 to 1,000 copies/ml); HHV-6, ≤1 plasmids/10 μl input (<100 copies/ml); HHV-8, 45 plasmids/10 μl input (4,500 copies/ml); JCV, 50 plasmids/10 μl input (4,500 copies/ml); and ERV-3, 45 plasmids/10 μl input (4,500 copies/ml).
of the mono-microbial agents, JCV was most frequent (10/20), and polymicrobial combination of two or three viruses in 25). Viruses were detected in 45/55 samples tested (monomicrobial in CMV, 25% versus 18%; EBV, 44% versus 31%). Overall, in CSF, brain tissues (CSF versus tissue positivity: JCV, 62% versus 42%; CMV, 100% versus 64%).

Comparing positivity rates of viruses in paired samples showed higher frequency in CSF than in tissue samples. Three of the most common viruses (JCV, CMV, HHV-6 were less common. Comparison of positivity rates of viruses in paired samples showed higher frequency in CSF than in brain tissues (CSF versus tissue positivity: JCV, 62% versus 42%; CMV, 25% versus 18%; EBV, 44% versus 31%). Overall, in CSF, viruses were detected in 45/55 samples tested (monomicrobial in 20 and polymicrobial combination of two or three viruses in 25). Of the mono-microbial agents, JCV was most frequent (10/20), followed by EBV and CMV (5 each).

In the polymicrobial group (n = 25 samples), JCV was again the most prevalent virus (24/25). In 19 individuals, co-infection with two viruses was seen in the CSF. In 18 of these, JCV was positive in combination with EBV (in 14 cases), CMV (in 3 cases), and HSV-1 (in 1 case). The remaining one case showed coexistence of EBV with HHV-6. In six individuals, co-infection with three viruses was detected. JCV was found in all six cases. In four, JCV co-infection was seen with EBV and CMV, while in the remaining two, JCV was seen in combination with CMV and VZV.

In tissue samples, viruses were detected in 37/55 samples tested (monomicrobial in 19, polymicrobial in 18). In 19 cases wherein a single virus was detected, JCV/EBV were seen in 7 cases each, CMV in four, and HHV-6 in one.

Of 18 individuals with polymicrobial infection, 17 had dual infection with two viruses, and in one individual co-infection with three viruses was seen. Of the 17 cases with dual infection, JCV was most frequently detected (15 of 17), co-infected with EBV in 8, CMV in 4, and HSV-1/VZV or HHV-6 in one case each. In the remaining two cases, combination of EBV with CMV or EBV with HHV-6 was detected. The single individual with triple infection had a combination of JCV with EBV and HHV-6.

None of the samples were positive for HHV-8, HSV-2, BK virus, or adenovirus. Among the 20 controls tested, a single CSF sample was positive for HHV-6, with a viral load of 33,400 copies/ml.

The EBV mean viral load in both CSF and tissue was very low (2.94 in CSF and 1.03 in tissue) compared to that of other viruses, such as JCV, CMV, VZV, HSV-1, and HHV-6. The data on the positivity rate for each virus in tissue and CSF with their mean viral load are shown in Table 1. The highest mean viral load in both CSF and brain was highest with JCV, followed by that for EBV and CMV. The PCR-positive rates and the mean viral loads for EBV, CMV, and JCV in paired CSF and tissue samples are shown in Table 2.

When the JCV, EBV, and CMV mean viral load levels in CSF (copies/ml) were compared with the respective mean viral load levels in brain tissue (copies/10,000 cells), the viral load levels were significantly (P < 0.001) high in the CSF for all three viruses, and these data are shown in Fig. 1. When the viral load levels in paired CSF and brain tissue samples were analyzed with Spearman’s rank (rho) test, there was a significant positive correlation in levels of JCV (r = 0.635, P = 0.01). JCV viral loads in both CSF (mean viral

---

### TABLE 1 Percentage positive and the mean viral loads detected in the paired CSF and tissue samples

<table>
<thead>
<tr>
<th>Virus</th>
<th>CSF (n = 55)</th>
<th>Tissue (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%) of positive samples</td>
<td>Viral load ( \log_{10} ) values (mean ± SD)</td>
</tr>
<tr>
<td>JCV</td>
<td>34 (62)</td>
<td>5.50 ± 1.78</td>
</tr>
<tr>
<td>EBV</td>
<td>24 (44)</td>
<td>2.94 ± 0.58</td>
</tr>
<tr>
<td>CMV</td>
<td>14 (25)</td>
<td>3.16 ± 1.27</td>
</tr>
<tr>
<td>VZV</td>
<td>2 (3.6)</td>
<td>7.00 ± 2.31</td>
</tr>
<tr>
<td>HSV-1</td>
<td>1 (1.8)</td>
<td>3.72</td>
</tr>
<tr>
<td>HHV-6</td>
<td>1 (1.8)</td>
<td>5.17</td>
</tr>
</tbody>
</table>

a The viral load expressed as \( \log_{10} \) copies/10,000 cells based on ERV-3 copies.

---

### TABLE 2 Total number of paired samples which were positive for viruses from various opportunistic infections

<table>
<thead>
<tr>
<th>Virus</th>
<th>Parameter</th>
<th>CSF (n = 55)</th>
<th>Tissue (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CM (n = 15)</td>
<td>TE (n = 17)</td>
</tr>
<tr>
<td>EBV</td>
<td>No. (%) of positive samples</td>
<td>6 (40)</td>
<td>7 (41)</td>
</tr>
<tr>
<td>Viral load (mean ± SD)</td>
<td>3.04 ± 0.51</td>
<td>2.87 ± 0.67</td>
<td>3.21 ± 0.55</td>
</tr>
<tr>
<td>CMV</td>
<td>No. (%) of positive samples</td>
<td>2 (13)</td>
<td>7 (41)</td>
</tr>
<tr>
<td>Viral load (mean ± SD)</td>
<td>2.94 ± 0.9</td>
<td>2.72 ± 0.65</td>
<td>4.70</td>
</tr>
<tr>
<td>JCV</td>
<td>No. (%) of positive samples</td>
<td>10 (67)</td>
<td>7 (41)</td>
</tr>
<tr>
<td>Viral load (mean ± SD)</td>
<td>5.28 ± 1.54</td>
<td>4.74 ± 0.30</td>
<td>4.60 ± 0.48</td>
</tr>
</tbody>
</table>

a All viral load values are expressed as \( \log_{10} \) copies/10,000 cells based on ERV-3 copies.

b The viral load expressed as \( \log_{10} \) copies/10,000 cells based on ERV-3 copies.
load, 7.96 log) and tissue (mean viral load, 6.06 log) samples were significantly high in confirmed cases of PML ($P$ value range from 0.003 to 0.013). In other opportunistic infections, the EBV viral load was found to be significantly high ($P = 0.031$) in CSF of cases of tuberculosis, compared to that for PML. The percentage positive and the mean viral load level of JCV in both CSF and tissue samples were significantly higher ($P < 0.002$) than those for CMV and EBV.

The EBV and CMV in-house quantitation assays were also validated with 6 samples for EBV and 4 samples for CMV received through the UKNEQAS program.

DNA from 24 JC-positive, 14 EBV-positive, and 9 CMV-positive samples was used for genotyping. All the 24 JCV strains were found to be genotype 2D. Out of 14 EBV strains, 8 were found to be EBV genotype 2, and 6 were EBV genotype 1. Out of 9 CMV strains, 8 were found to be genotype gB1, and 1 was found to be genotype gB2.

One of the important findings in our study is that viruses like EBV, CMV, and JC virus were commonly found in the HIV-infected individuals who succumbed to various bacterial, fungal, or parasitic infections. In an earlier study from India, 147 CSF samples from various neurological conditions in non-HIV-infected individuals using real-time multiplex PCR had reported the presence of multiple viruses in a single sample, with EBV (34%) being most frequent (15).

A study from Italy of herpesvirus DNA in CSF of AIDS patients with CNS disease found one or more of the following viruses in 26 of 49 patients (53%); CMV in 16 (33%), EBV in 13 (27%), HHV-6 in 2 (4%), HHV-7 in 1 (2%), and HHV-8 in 1 (2%). CMV was associated with the occurrence of encephalitis and peripheral neuropathy and EBV with primary CNS lymphoma (16). The study also reported that 7 (14%) patients had DNA for more than one herpesvirus (16). In another study from Italy employing nested PCR on CSF in HIV-infected individuals, coinfections with more than one virus in CSF was reported in 181 out of 500 patients (17).

In our study, only one (5%) of the 20 CSF samples of the control group was positive for HHV-6. These results show that the assays we had used in this study are very robust and specific. The higher proportion of positivity in our study population may be due to sampling in the late stage of AIDS. Significantly, a high viral load of JCV in PML suggests that a critical threshold may be required for clinical manifestation with disease. The detection of multiple viruses in a given sample reflects the biological phenomena of transactivation, which needs to be explored further.

In our study, we also examined the genotypes of the three common viruses seen in our population. A study that reported on the molecular characterization of JC virus in Brazilian HIV-infected individuals with and without PML reported JC virus genotypes 2 (32.7%), 1 (26.5%), and 3 (23.5%) in their population from CSF and urine samples. They also reported that JCV genotype 1 showed a strong association with PML ($P < 0.0001$), while JCV genotype 3 had an inverse association with PML (18). Agostini et al. had found JC virus genotype 2B to be more frequent (3.6%) in brain tissues of patients with PML than in urine from controls (5.9%), and genotype 2B predicts the highest risk of PML disease in HIV-infected individuals (19). In our study population, we observed only a single type of JC genotype, i.e., 2D in CSF and brain tissues samples of both cases with and without PML.
Several studies on EBV genotypes in EBV-related diseases like Hodgkin’s disease, Burkitt’s lymphoma, Hodgkin’s lymphoma, and oral hairy leukoplakia had reported a higher percentage of EBV genotype 1, with 56% (20), 58% (21), 82% (22), and 93% (23), respectively, and in the same clinical conditions the EBV genotype 2 frequencies were 7% (23), 9% (22), 13% (20), and 23% (21), respectively. In contrast to these studies, our study population had more or less the same frequency of EBV genotype 2 (57%) and EBV genotype 1 (43%). A PCR-based restriction fragment length polymorphism (RFLP) study from India (Chennai) in patients with CMV disease reported CMV gB2 and gB3 in 53.8 and 46.1% of the patients studied, respectively (24). Our data showed a high frequency of CMV genotype gB1 (89%), followed by gB2 genotype (11%).

This study emphasizes the utility of multiplex real-time PCR assays in the simultaneous detection of multiple viral agents. The presence of multiple viruses in the same sample suggests one infectious agent leading to the reactivation of several other latent viruses and is of clinical relevance, essential for determining patient management.

ACKNOWLEDGMENTS
We gratefully acknowledge the funding received from the Department of Biotechnology (Government of India) for the study.

The authors also thank the Human Brain Tissue Repository (HBTR; or Brain Bank), National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, for providing the CSF and brain tissue samples for the study.

Data presented in the manuscript form part of the Ph.D. thesis of Jaiprasath Sachithanandham.

The authors report that they have no conflicts of interests.

REFERENCES