

Human Herpesvirus 6 Detection in Alzheimer's Disease Cases and Controls across Multiple Cohorts

Highlights

- RNA-seq data from two Alzheimer's disease (AD) cohorts were screened for 118 viruses
- DNA from 711 AD and control brains was analyzed for PCR reactivity to HHV-6A and/or HHV-6B
- HHV-6 demonstrated little specificity to AD brains over controls by either method
- These complementary methods do not support strong association between HHV-6 and AD

Authors

Mary Alice Allnutt, Kory Johnson, David A. Bennett, ..., Sonja W. Scholz, Philip L. De Jager, Steven Jacobson

Correspondence

jacobsons@ninds.nih.gov

In Brief

Allnutt et al. screen three independent Alzheimer's disease (AD) cohorts for pathogens, including 118 human viruses, using RNA-seq and PCR from 711 AD and non-AD control brains. No differences in viral detection between AD and non-AD controls are observed.



Human Herpesvirus 6 Detection in Alzheimer's Disease Cases and Controls across Multiple Cohorts

Mary Alice Allnutt,¹ Kory Johnson,² David A. Bennett,³ Sarah M. Connor,⁴ Juan C. Troncoso,⁵ Olga Pletnikova,⁵ Marilyn S. Albert,⁶ Susan M. Resnick,⁷ Sonja W. Scholz,^{6,8} Philip L. De Jager,⁴ and Steven Jacobson^{1,9,*}

¹Viral Immunology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20814, USA

²Bioinformatics Section, Information Technology & Bioinformatics Program, Division of Intramural Research (DIR), National Institute of Neurological Disorders and Stroke/National Institute of Health, Bethesda, MD 20814, USA

³Alzheimer Disease Center, RUSH University, Chicago, IL 60612, USA

⁴Center for Translational & Computational Neuroimmunology, Department of Neurology, Columbia University Medical Center, New York, NY 10032, USA

⁵Department of Pathology (Neuropathology), Johns Hopkins University Medical Center, Baltimore, MD 21205, USA

⁶Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

⁷Laboratory of Behavioral Neuroscience, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA

⁸Neurodegenerative Diseases Research Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20814, USA

⁹Lead Contact

*Correspondence: jacobsons@ninds.nih.gov

<https://doi.org/10.1016/j.neuron.2019.12.031>

SUMMARY

The interplay between viral infection and Alzheimer's disease (AD) has long been an area of interest, but proving causality has been elusive. Several recent studies have renewed the debate concerning the role of herpesviruses, and human herpesvirus 6 (HHV-6) in particular, in AD. We screened for HHV-6 detection across three independent AD brain repositories using (1) RNA sequencing (RNA-seq) datasets and (2) DNA samples extracted from AD and non-AD control brains. The RNA-seq data were screened for pathogens against taxon references from over 25,000 microbes, including 118 human viruses, whereas DNA samples were probed for PCR reactivity to HHV-6A and HHV-6B. HHV-6 demonstrated little specificity to AD brains over controls by either method, whereas other viruses, such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV), were detected at comparable levels. These direct methods of viral detection do not suggest an association between HHV-6 and AD.

INTRODUCTION

Alzheimer's disease (AD) is a neurological disease characterized by extensive accumulation of the amyloid-beta (A β) peptide, deterioration of brain structures, neuroinflammation, and ultimately dramatic cognitive deficits (Holmes, 2013; Regen et al., 2017). The primary models of AD focus on amyloid pathology (Brody et al., 2017; Fessel, 2018; Moir and Tanzi, 2019; Walsh

et al., 2002), although there has been renewed interest in potential infectious triggers of AD (Eimer et al., 2018; Goldschmidt-Clermont et al., 2019; Qin and Li, 2019; Readhead et al., 2018). Many pathogens have been associated with the disease, including *Chlamydia pneumoniae* (Balin and Hudson, 2014) and spirochaetes (Miklossy, 2015), but most frequently, human herpesviruses such as human herpesvirus 6A (HHV-6A), HHV-6B, and herpes simplex virus 1 (HSV-1) have been linked to AD pathogenesis (Ball, 1982; Haas and Lathe, 2018; Itzhaki et al., 1997; Lin et al., 2002; Liu et al., 2018; Rizzo et al., 2019; Romeo et al., 2019a, 2019b; Wozniak et al., 2009).

Both HSV-1 and HHV-6 are active within the CNS, with HSV-1 causing HSV encephalitis and HHV-6, particularly HHV-6A, being linked to various CNS diseases, including multiple sclerosis, epilepsy, and encephalitis (Leibovitch and Jacobson, 2018; Piacentini et al., 2014). Studies comparing AD and non-AD controls have detected increased HHV-6 DNA (Lin et al., 2002), as well as increased co-localization of HSV-1 DNA with A β plaques (Wozniak et al., 2009) in AD brains. Compared to non-AD controls, AD samples have been found to have decreased HHV-6 IgG titer in blood (Haas and Lathe, 2018) and increased risk of AD development in the presence of HSV-1 in the brain (Itzhaki et al., 1997) or plasma (Lövheim et al., 2018). In addition, several groups have identified overlap between AD genetic risk factors and genes affected by viral infection, such as a receptor involved in spreading HSV-1 (Liu et al., 2018) and a human leukocyte antigen (HLA) subtype associated with increased susceptibility to HHV-6A infection (Rizzo et al., 2019).

Two recent publications (Eimer et al., 2018; Readhead et al., 2018) in particular have brought a renewed focus on the role of HHV-6 and HSV-1 in the pathogenesis of AD. Extensive analysis of RNA sequencing (RNA-seq) and whole-genome sequencing data available through several independent brain banks were used to identify associations between HHV-6A and/or HHV-7



Table 1. Disease Demographics and Repositories Analyzed

Mount Sinai Brain Bank (MSBB)	Religious Orders Study/Memory and Aging Project (ROSMAP)			Johns Hopkins Brain Resource Center (JHBRC)		
RNA-seq n = 301 ^a	RNA-seq n = 600	DNA ddPCR n = 364	DNA ddPCR n = 344			
definite AD ^b	135	definite AD ^b	173	AD	267	AD
probable AD ^b	42	probable AD ^b	207	AD	267	multiple system atrophy (MSA)
possible AD ^b	38	possible AD ^b	62	non-AD controls	97	progressive supranuclear palsy (PSP)
non-AD controls ^b	86	non-AD controls ^b	158	non-AD controls	97	non-AD controls
						243
						12
						20
						69

Two data types were analyzed from three different brain banks: (1) RNA-seq data from AD and non-AD control brains were analyzed via PathSeq from both MSBB (n = 301) and ROSMAP (n = 600) cohorts, whereas (2) DNA extracted from AD and non-AD control brains from both ROSMAP (n = 364) and JHBRC (n = 344) was assessed for PCR reactivity.

^aUp to four brain regions screened per individual (BM10, BM22, BM36, BM44). Total number of specimens = 1,027.

^bAD classification coding: neuropathology categories as measured by CERAD (Wang et al., 2018; Bennett et al., 2012a, 2012b)

and AD, citing an increase in both viral RNA and DNA in AD brains over controls (Readhead et al., 2018). Furthermore, the authors observed that host genes that are upregulated by viral infection extensively overlapped with AD risk genes (Readhead et al., 2018). Supporting the association between HHV-6, HSV-1, and AD, another study suggested that the aggregation of A β could be stimulated by the presence of either HHV-6 or HSV-1 (Eimer et al., 2018). Amyloid plaques formed around these viruses, providing an antiviral protective effect both *in vitro* and in a 5xFAD mouse model (Eimer et al., 2018). Collectively, these studies provide a mechanistic framework for the role of herpesviruses in AD.

Given the renewed interest in infectious triggers in AD with the recent focus on HHV-6 combined with our long-standing experience in studies on the association of neurologic disorders and this virus, e.g., multiple sclerosis (Leibovitch et al., 2019; Virtanen et al., 2014), epilepsy (Bartolini et al., 2018), glioblastomas (Lin et al., 2016), and post-transplant encephalitis (Yao et al., 2009), it was of interest to us to explore the observation that HHV-6A and/or HHV-6B could be involved in the pathogenesis of AD. We approached this question in two ways: (1) a re-analysis of the bioinformatic data using RNA-seq datasets from the Mount Sinai Brain Bank (MSBB) and the Religious Orders Study/Memory and Aging Project (ROSMAP) and (2) a direct interrogation of brain material for HHV-6A and HHV-6B PCR reactivity from AD patients and controls from which DNA had been extracted and that we had access to from ROSMAP (Bennett et al., 2012a, 2012b) and the Johns Hopkins Brain Resource Center (JHBRC), which includes brain autopsies from the NIA Baltimore Longitudinal Study of Aging (Blauwendraat et al., 2019). The Broad Institute PathSeq tool, which screens over 25,000 microbes, including 118 human viruses, was used for RNA-seq analysis (Walker et al., 2018). PCR detection for HHV-6A and HHV-6B utilized a digital droplet PCR (ddPCR) platform, which is a highly sensitive and precise novel PCR methodology that we have used extensively (Leibovitch et al., 2014). The findings of this study, using the complementary methods of PathSeq and ddPCR on samples from three independent repositories, demonstrated little difference in viral detection between AD and non-AD controls.

RESULTS

Disease Demographics and Repositories Analyzed

RNA-seq datasets were available from both MSBB and ROSMAP using the AMP-AD Knowledge Portal on SYNAPSE (MSBB synapse ID: syn3157743; ROSMAP: syn3388564) (Table 1) and were filtered into the PathSeq pipeline from the Broad Institute to screen for pathogens (Walker et al., 2018). In the MSBB repository, RNA-seq data were available from 301 individuals, from which up to four different brain regions from each individual were screened for a total of 1,027 specimens (Table 1). The four brain regions included Brodmann area 10 (BM10), the anterior prefrontal cortex; Brodmann area 22 (BM22), the superior temporal gyrus; Brodmann area 36 (BM36), the parahippocampal gyrus; and Brodmann area 44 (BM44), the inferior frontal gyrus (Readhead et al., 2018; Wang et al., 2018). From the ROSMAP repository, RNA-seq data were available from 600 brain samples from the dorsolateral prefrontal cortex (Bennett et al., 2012a, 2012b) (Table 1). For both MSBB and ROSMAP repositories, brains were classified with respect to AD disease status using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropathology classification, which ranks individuals in categories of "No AD," "Possible AD," "Probable AD," and "Definite AD" (Table 1) (Bennett et al., 2012a, 2012b; Wang et al., 2018). In addition to RNA-seq data, we were fortunate to be able to obtain extracted DNA for ddPCR analysis from 364 brains also from the ROSMAP brain repository (although DNA from these brains did not have corresponding RNA-seq information). DNA extracted from an additional 344 brains was also made available to us from a third brain repository, the JHBRC, comprising 243 AD brains and 101 non-AD controls (Blauwendraat et al., 2019). These DNA samples were extracted for genetic analysis of neurodegenerative diseases in a well-characterized clinicopathological cohort (Blauwendraat et al., 2019). RNA-seq data were also not available for this cohort.

Detection of HHV-6 in MSBB and ROSMAP RNA-Seq Datasets

RNA-seq data from the MSBB cohort were available from multiple brain regions for 301 total individuals. Clinical data for each

individual included neocortical plaque density across five regions (number of plaques/mm²) and clinical dementia rating (CDR) score (Wang et al., 2018), which stratifies clinical symptoms of dementia by severity. When individuals were arranged by a compounded Plaque * CDR score (disease severity score), it was possible to easily visualize the correlation between PathSeq results and clinical and neuropathological disease (Figure 1A). The PathSeq score, based on the number of reads that align with taxon references, indicates the amount of evidence that a taxon is present in a given sample (Walker et al., 2018). These scores, defined by the Broad Institute (https://software.broadinstitute.org/gatk/documentation/tooldocs/current/org_broadinstitute_hellbender_tools_spark_pathseq_PathSeqScoreSpark.php), are used to “detect and quantify microbe abundance. Alignments with sufficient identity score (e.g. 90% of read length) estimate read counts and the relative abundance of microorganisms present in the sample at each level of the taxonomic tree... Reads with a single valid alignment add a score of 1 to the corresponding species or strain. For reads with N hits, a score of 1/N is distributed to each organism. Scores are totaled for each taxon by summing the scores across all reads and the scores of any descendent taxa.” Pathogen load is correlated with score as score is based on RNA read counts.

PathSeq scores for HHV-6 within the MSBB cohort were fairly low, with the highest score being 33.5 and most scores remaining between 0 and 10. All of the positive reads for HHV-6 fell within samples from just four out of 301 individuals, with HHV-6 found in multiple brain regions for two of those individuals (Figure 1B). There appears to be little difference in the frequency of detection between HHV-6A and HHV-6B. Of the four individuals in which any HHV-6 signal was detected, three out of four had both HHV-6A and HHV-6B, and one individual (Person 1) had only HHV-6B with a low PathSeq score of 1.0 (and the lowest disease severity score) (Figures 1A and 1B). Person 2 had a modest PathSeq score of 33.5 in the BM22 brain region for HHV-6B, and Person 3 had a likewise modest PathSeq score of 23.5 for HHV-6A also in BM22 (Figures 1A and 1B). There was no correlation between disease severity and PathSeq score (Figure 1A; R² = 0.0016).

In addition to the MSBB cohort, RNA-seq data were available from ROSMAP. The dataset included 600 individuals of varying AD classifications (Table 1). Similar to MSBB, PathSeq analysis demonstrated a low frequency of HHV-6 detection (with low PathSeq scores) in ROSMAP, with HHV-6A detected in only two brains in this cohort (Figure 1C). No HHV-6B was detected. Both ROSMAP and MSBB classified samples according to CERAD neuropathology guidelines (Bennett et al., 2012a, 2012b; Wang et al., 2018). As shown in Figure 1D, HHV-6A was detected in only one out of 173 brains with definite AD and only one out of 158 non-AD, aged controls in the ROSMAP dataset (Figure 1D). In the MSBB cohort, HHV-6A and HHV-6B were detected in three out of 135 brains with definite AD, and HHV-6B was detected in an additional one out of 42 brains with probable AD (Figure 1D).

Specificity of Viral Detection in MSBB and ROSMAP RNA-Seq Datasets

In addition to HHV-6A and HHV-6B, several other CNS-associated viruses were also detected by PathSeq in both MSBB

and ROSMAP (Figure 2). These included John Cunningham virus (JCV) (Bartsch et al., 2019), human immunodeficiency virus 1 (HIV) (Nath, 2015), Epstein-Barr virus (EBV) (Leibovitch and Jacobson, 2018), and cytomegalovirus (CMV) (Bookstaver et al., 2017), although PathSeq scores for each were all less than seven (Figure S1).

The frequency of detection for any virus was extremely low and was not significantly different between AD (nor between sub-classifications of AD) and non-AD brains (Figure 2; Table S1). For example, in the MSBB and ROSMAP brain cohorts, 6.0% and 0.9%, respectively, of brain specimens with definite, probable, or possible AD contained EBV reads compared with 5.8% and 1.3% of brains from non-AD controls (Figure 2). With a particular focus on HHV-6 (including both HHV-6A and HHV-6B) in the MSBB and ROSMAP brain cohorts, 3.3% and 0.2%, respectively, of brain specimens with definite, probable, or possible AD contained HHV-6 reads compared with 0% and 0.6% of brains from non-AD, aged controls (Figure 2). Again, no statistical difference was found in the frequency of any one virus between diseased and control brains, and there was no evidence for virus specificity.

HHV-6 Screening Using ddPCR

To further explore the association between HHV-6 and AD, DNA from available brain sections were subjected to PCR amplification of HHV-6A- and HHV-6B-specific regions (Leibovitch et al., 2014). A total of 708 brain sections were used from two brain repositories, including 344 brain brains from a JHBRC collection and 364 brains from the ROSMAP brain bank consisting of AD and non-AD controls (Table 1). The frequency of detection of HHV-6 was low in both cohorts (Figure 3). Only one individual in the ROSMAP non-AD control cohort was positive for HHV-6A (Figure 3). In addition, the frequency of HHV-6B detection was not significantly different between AD and non-AD controls for either the ROSMAP (χ^2 p = 0.55) or JHBRC (χ^2 p = 0.76) cohorts (Figure 3). The magnitude of HHV-6 PCR reactivity was also low and did not vary between AD and non-AD controls for either ROSMAP (p = 0.39) or JHBRC (p = 0.40), with the majority of positive brains demonstrating a viral load of fewer than 600 copies of HHV-6/10⁶ cells (Figure 3). Of interest was the observation that within the 708 brain samples tested for HHV-6 PCR reactivity, five brains had PCR reactivity at approximately one copy per cell (Figure S2A), suggesting that these individuals had chromosomally integrated HHV-6 (ciHHV-6) (Flamand, 2018). CiHHV-6A was detected in three brains from the ROSMAP cohort, one with AD and two non-AD controls, whereas ciHHV-6B was detected in two AD brains from JHBRC (Figure S2B). This observation of ciHHV-6 in five out of 708 (0.71%) brains is consistent with the known prevalence of ciHHV-6, which is present in less than 1% of the healthy human population (Flamand, 2018).

DISCUSSION

Although many studies have reported evidence of an association between HHV-6, HSV-1, and AD (Eimer et al., 2018; Haas and Lathe, 2018; Itzhaki et al., 1997; Lin et al., 2002; Liu et al., 2018; Lövheim et al., 2018; Readhead et al., 2018; Rizzo et al., 2019; Romeo et al., 2019b; Wozniak et al., 2009), these results

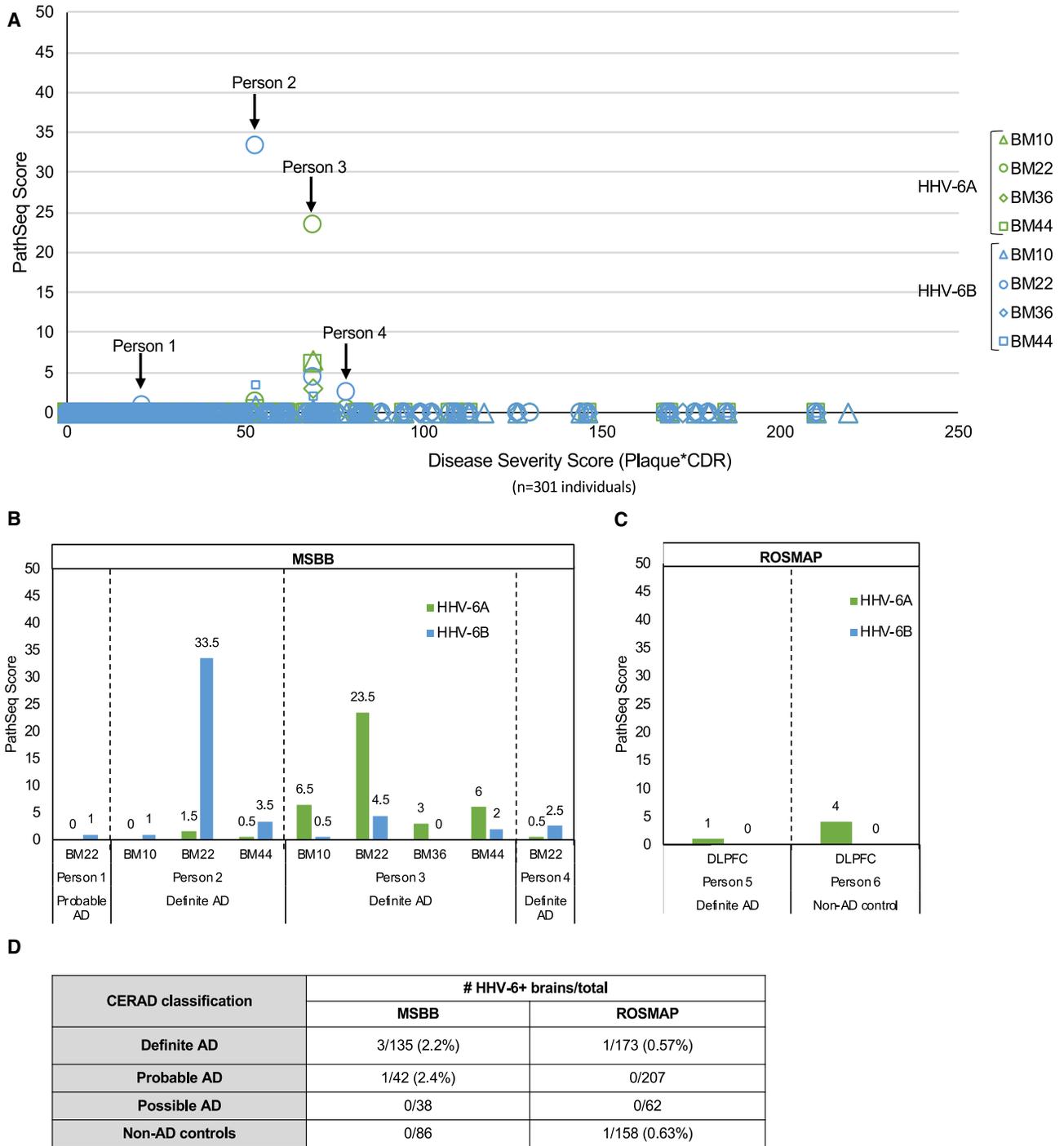


Figure 1. Detection of HHV-6 in MSBB and ROSMAP RNA-Seq Datasets

(A) HHV-6A and -6B PathSeq scores are displayed in order of increasing disease severity score (calculated as neuritic plaque density * Clinical Dementia Rating score).

(B) HHV-6 was detected in nine specimens from four individuals in the MSBB cohort, with varying PathSeq scores.

(C) HHV-6A was detected in two individuals in the ROSMAP cohort, both with extremely low PathSeq scores, whereas no HHV-6B was detected.

(D) Brains with HHV-6 in one or more brain regions are classified by CERAD neuropathology, with HHV-6 detected in individuals with definite and probable AD in addition to one non-AD control brain.

BM10, Brodmann area 10, anterior prefrontal cortex; BM22, Brodmann area 22, superior temporal gyrus; BM36, Brodmann area 36, parahippocampal gyrus; BM44, Brodmann area 44, inferior frontal gyrus; DLPFC, dorsolateral prefrontal cortex.

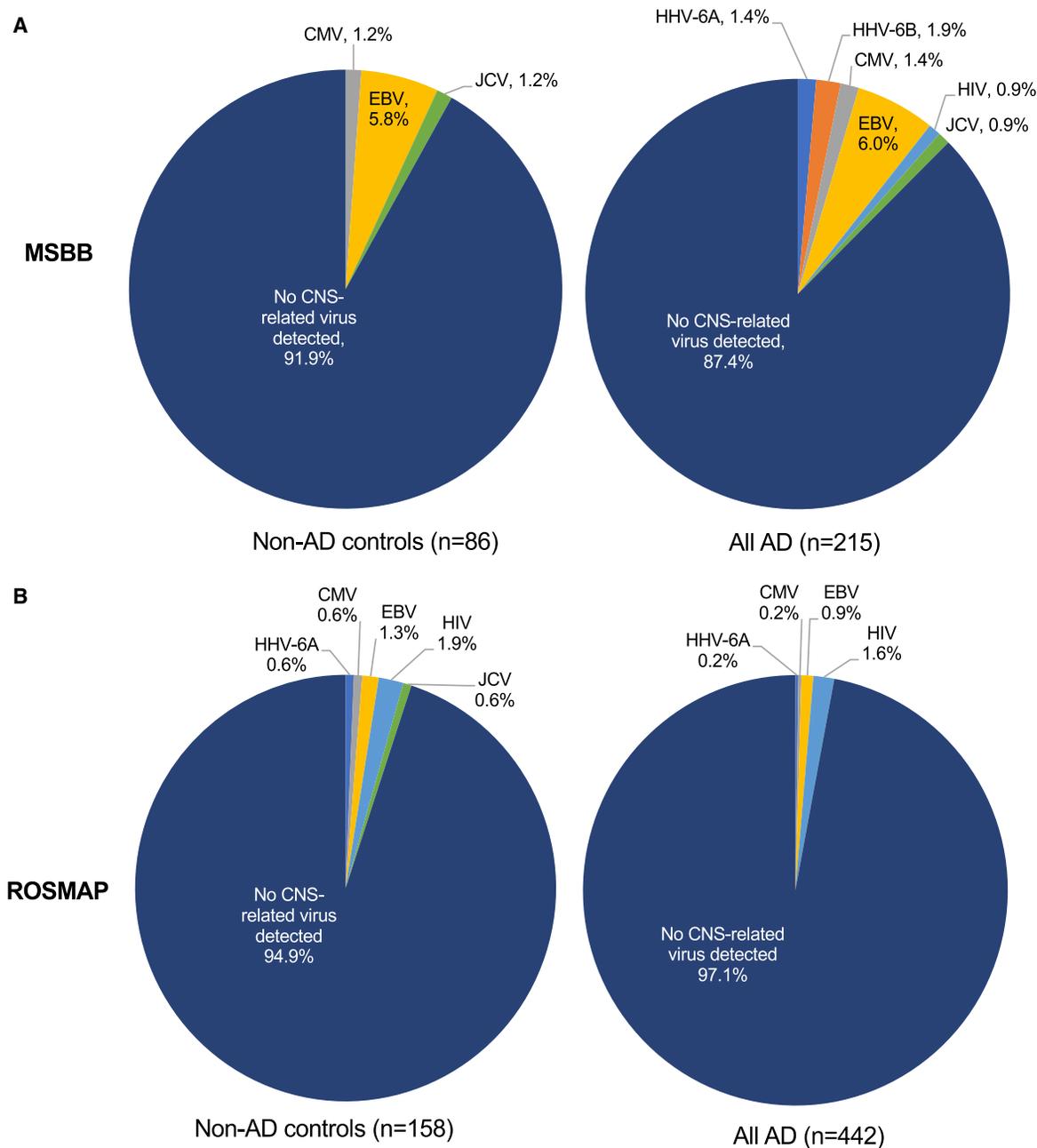


Figure 2. CNS-Related Virus Detection across Both MSBB and ROSMAP

(A and B) All CNS-related viruses detected by PathSeq analysis were distributed across AD classifications in both (A) MSBB and (B) ROSMAP. All CNS-related viruses detected in brains from the definite, probable, and possible AD neuropathology categories are compared to non-AD controls for both cohorts. See also Figure S1.

CMV, cytomegalovirus; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; JCV, John Cunningham virus.

have been tempered by contemporaneous studies that found little or no association (Agostini et al., 2016; Hemling et al., 2003) and an apparent lack of specificity in the viruses that are identified. Other viruses, such as EBV, CMV, and Kaposi's sarcoma-associated herpesvirus (KSHV), have also been associated with AD development and progression (Carbone et al., 2014; Lövheim et al., 2018; Qin and Li, 2019; Talwar et al., 2019)

through techniques such as PCR analysis of the brain or plasma (Carbone et al., 2014; Lövheim et al., 2018) and bioinformatic analysis (Talwar et al., 2019).

The breadth of viral species that have been associated with AD is also reflected in our analysis of the MSBB and ROSMAP RNA-seq datasets. Transcripts from viruses such as EBV and CMV were detected at frequencies and PathSeq scores comparable

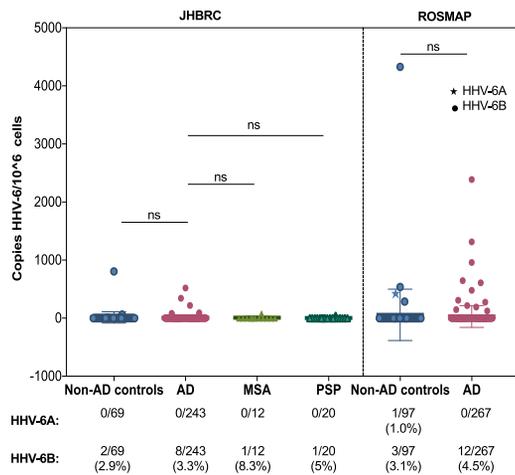


Figure 3. HHV-6 Detection via ddPCR across Disease Statuses

The HHV-6B viral load, in copies per 10^6 cells, is compared between AD and non-AD samples in both the ROSMAP and JHBRC cohorts. Viral load did not differ significantly across groups (one-way ANOVA $p = 0.41$). The frequency of detection of HHV-6A and HHV-6B did not differ between AD and non-AD samples ($\chi^2 p = 0.81$), with frequencies all remaining well below 10% in each disease cohort. Data are represented as mean \pm SD.

to HHV-6, and our study did not suggest major differences in HHV-6 expression between AD and non-AD control brains. Moreover, in the MSBB cohort, all instances of HHV-6 detection occurred in brains from only four (out of 301) individuals and two (out of 605) individuals in the ROSMAP cohort. Even among brains with detectable HHV-6 RNA, the PathSeq scores were low. Although PathSeq scores of 0.5 or 1 here are scored as positive, a higher score indicates more robust evidence that a taxon is present based on the number of reads that aligned to taxon references. The Broad Institute that developed the PathSeq tool in their genome analysis toolkit (GATK) has reported positive reads in the thousands (Walker et al., 2018). Indeed, PathSeq was recently used to identify viruses from brains obtained from two cases with fatal encephalopathies (one with JCV and the other with Dengue virus) in which PathSeq scores were 5,000 or more (Johnson et al., 2019; Reoma et al., 2019). However, analysis of bulk RNA-seq project data that aims for traditional gene expression profiling and does not include pathogen detection and screening as a project aim can render misleading, false-negative results. The location and the time at which RNA was collected and the depth of sequencing in conjunction with a biased transcriptome background can each lead to reduced viral detection.

Although the DNA sequence data from MSBB and ROSMAP were not available for download at the time of analysis to support these results, ddPCR of DNA from both the ROSMAP and JHBRC cohorts did not reveal any difference in frequency of HHV-6 detection between AD and non-AD control groups. The frequency of HHV-6B detection in each brain bank remained very low, regardless of disease status. In addition, only one sample out of the 708 tested was positive for HHV-6A DNA, and this sample fell into the non-AD control category, again not supporting any significant associations with HHV-6A and AD. Whereas

previous studies have found higher frequencies of HHV-6 DNA in brains, this frequency varies widely across the literature, with percentages of detection anywhere between 2% and 70% (Cermelli et al., 2003; Chan et al., 2001; Chapenko et al., 2016; Hemling et al., 2003; Lin et al., 2002; Wipfler et al., 2018). Such discrepancies could be related to differences in PCR methodologies, different PCR primer and pair sequences, and different brain regions analyzed. The majority of these studies used DNA collected from multiple brain regions, whereas the DNA in the present study were collected from the dorsolateral prefrontal cortex.

These results add to the controversial nature of the evidence for an association between HHV-6 and AD. However, our results do not preclude the validity of the associations found by previous studies. Whereas in the present study we directly analyzed the frequency of the virus in AD as compared to control brains by PCR, other authors used more indirect measures, such as anti-HHV-6 IgG levels in serum (Agostini et al., 2016; Haas and Lathe, 2018) or the larger network of genes that relate HHV-6 to AD risk loci (Readhead et al., 2018; Rizzo et al., 2019). In addition, RNA-seq analysis methods can differ at any stage of processing of raw read files, including quality inspection, adaptor clipping, quality trimming, rRNA read filtering, reference mapping, and feature enumeration. Despite the source data being exactly the same, a different method or rule at any single step can produce different end results. Analyses of the same MSBB and ROSMAP RNA-seq data may vary in the length of nucleotide sequence (kmer) used, the method to derive the dictionary of kmers that differentiate host reference sequences from non-host, and, in the end, how those dictionaries compare between methods. Currently, there is no standard generalized kmer library for this purpose used across authors. Rather, they are author devised by methods and rules they chose, contributing to the variation in pathogen detection between analyses of the same datasets.

Our results suggest that if viruses (and HHV-6 in particular) do play a role in AD pathogenesis, then the agents may no longer be present in a form that can be PCR amplified or sufficiently expressed. Rather, these virus(es) may be associated with an earlier “triggering” event or be present at copy numbers below the limit of laboratory detection. Early triggering events may include nucleation of amyloid beta ($A\beta$) that has recently been reported to have antimicrobial activity against bacteria, fungi, and viruses, including HHV-6 (Bourgade et al., 2015, 2016; Eimer et al., 2018; Kumar et al., 2016; Soscia et al., 2010; Spitzer et al., 2016; White et al., 2014). Nucleation of $A\beta$ enveloping, for example, a herpesvirus virion (Eimer et al., 2018) may be important for AD development but would not necessarily result in the detection of the complete virus, thus providing a potential explanation for the lack of easily identifiable pathogen in the brains of AD patients.

The findings of this study, using the complementary methods of PathSeq and ddPCR on samples from three independent repositories, do not support an association between HHV-6 and AD but also do not rule it out. Clearly, the observations in this report need to be extended to larger collaborative studies using multiple bioinformatic analysis pipelines and laboratory-based tools. Future studies on the role of the HHV-6 virus in the progression and development of AD should focus on the

mechanisms by which this virus could play an important role while remaining largely undetected in large-scale analyses of brain material.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- [KEY RESOURCES TABLE](#)
- [LEAD CONTACT AND MATERIALS AVAILABILITY](#)
- [EXPERIMENTAL MODEL AND SUBJECT DETAILS](#)
- [METHOD DETAILS](#)
 - [Screening for Virus Using RNA-Seq Data](#)
 - [Screening for Virus Using Droplet Digital PCR \(ddPCR\)](#)
- [QUANTIFICATION AND STATISTICAL ANALYSIS](#)
 - [Statistical Analysis of PathSeq Results](#)
 - [Quantification of ddPCR Results](#)
 - [Statistical Analysis of ddPCR Results](#)
- [DATA AND CODE AVAILABILITY](#)

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.neuron.2019.12.031>.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program, National Institute of Neurological Disorders and Stroke, National Institutes of Health.

DNA samples from ROSMAP were provided by the Rush Alzheimer's Disease Center and can be requested along with data from <https://www.radc.rush.edu>. DNA from the JHBRC cohort was provided by Dr. Sonja Scholz in collaboration with the Johns Hopkins Department of Neurology. This research was supported in part by the Intramural Research Program of the NIH National Institute of Neurological Disorders and Stroke (project number: ZIA-NS003154). We would like to thank the NIA Baltimore Longitudinal Study of Aging for contributing tissue samples to the Johns Hopkins Alzheimer's Disease Research Center. The results published here are in whole or in part based on data obtained from the AMP-AD Knowledge Portal (<https://doi.org/10.7303/syn2580853>; <https://doi.org/10.1038/s41593-018-0154-9>). Study data were provided by the Rush Alzheimer's Disease Center, Rush University Medical Center, Chicago. Data collection was supported through funding by NIA grants P30AG10161, R01AG15819, R01AG17917, R01AG30146, R01AG36836, U01AG32984, U01AG46152, and U01AG61356, in addition to JHU ADRC (P50AG05146), the Illinois Department of Public Health, the Translational Genomics Research Institute, and the Intramural Research Program of the National Institute on Aging, NIH. These data were generated from postmortem brain tissue collected through the Mount Sinai VA Medical Center Brain Bank and were provided by Dr. Eric Schadt from Mount Sinai School of Medicine.

AUTHOR CONTRIBUTIONS

M.A.A., S.J., and K.J. contributed to the study's overall conception, design, and interpretation. M.A.A. and S.J. wrote the manuscript, while M.A.A. and K.J. created the figures with input from S.J. K.J. conducted analysis of RNA-seq data, while M.A.A. conducted PCR analysis of all DNA samples. S.W.S. provided DNA samples from JHBRC, and P.L.D. provided DNA samples from ROSMAP. S.M.C. performed quantification and preparation of DNA samples from ROSMAP. D.A.B. is the principal investigator behind ROSMAP. J.C.T. and O.P. are the neuropathologists from the Hopkins Brain Resource Center who performed the pathological evaluation of the included samples

from JHBRC; M.S.A. and S.M.R. recruited many of the AD cases and controls that are in the JHBRC.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: September 23, 2019

Revised: November 11, 2019

Accepted: December 26, 2019

Published: January 23, 2020

REFERENCES

- Agostini, S., Mancuso, R., Baglio, F., Cabinio, M., Hernis, A., Guerini, F.R., Calabrese, E., Nemni, R., and Clerici, M. (2016). Lack of evidence for a role of HHV-6 in the pathogenesis of Alzheimer's disease. *J. Alzheimers Dis.* *49*, 229–235.
- Balin, B.J., and Hudson, A.P. (2014). Etiology and pathogenesis of late-onset Alzheimer's disease. *Curr. Allergy Asthma Rep.* *14*, 417.
- Ball, M.J. (1982). "Limbic predilection in Alzheimer dementia: is reactivated herpesvirus involved?". *Can. J. Neurol. Sci.* *9*, 303–306.
- Bartolini, L., Piras, E., Sullivan, K., Gillen, S., Bumbut, A., Lin, C.M., Leibovitch, E.C., Graves, J.S., Waubant, E.L., Chamberlain, J.M., et al. (2018). Detection of HHV-6 and EBV and Cytokine Levels in Saliva From Children With Seizures: Results of a Multi-Center Cross-Sectional Study. *Front. Neurol.* *9*, 834.
- Bartsch, T., Remppe, T., Leypoldt, F., Riedel, C., Jansen, O., Berg, D., and Deuschl, G. (2019). The spectrum of progressive multifocal leukoencephalopathy: a practical approach. *Eur. J. Neurol.* *26*, 566–e41.
- Bennett, D.A., Schneider, J.A., Arvanitakis, Z., and Wilson, R.S. (2012a). Overview and findings from the religious orders study. *Curr. Alzheimer Res.* *9*, 628–645.
- Bennett, D.A., Schneider, J.A., Buchman, A.S., Barnes, L.L., Boyle, P.A., and Wilson, R.S. (2012b). Overview and findings from the rush Memory and Aging Project. *Curr. Alzheimer Res.* *9*, 646–663.
- Blauwendraat, C., Pletnikova, O., Geiger, J.T., Murphy, N.A., Abramzon, Y., Rudow, G., Mamais, A., Sabir, M.S., Crain, B., Ahmed, S., et al. (2019). Genetic analysis of neurodegenerative diseases in a pathology cohort. *Neurobiol. Aging* *76*, 214.e1–214.e9.
- Bookstaver, P.B., Mohorn, P.L., Shah, A., Tesh, L.D., Quidley, A.M., Kothari, R., Bland, C.M., and Weissman, S. (2017). Management of Viral Central Nervous System Infections: A Primer for Clinicians. *J. Cent. Nerv. Syst. Dis.* *9*, 1179573517703342.
- Bourgade, K., Gameau, H., Giroux, G., Le Page, A.Y., Bocti, C., Dupuis, G., Frost, E.H., and Fülöp, T., Jr. (2015). β -Amyloid peptides display protective activity against the human Alzheimer's disease-associated herpes simplex virus-1. *Biogerontology* *16*, 85–98.
- Bourgade, K., Le Page, A., Bocti, C., Witkowski, J.M., Dupuis, G., Frost, E.H., and Fülöp, T., Jr. (2016). Protective Effect of Amyloid- β Peptides Against Herpes Simplex Virus-1 Infection in a Neuronal Cell Culture Model. *J. Alzheimers Dis.* *50*, 1227–1241.
- Brody, D.L., Jiang, H., Wildburger, N., and Esparza, T.J. (2017). Non-canonical soluble amyloid-beta aggregates and plaque buffering: controversies and future directions for target discovery in Alzheimer's disease. *Alzheimers Res. Ther.* *9*, 62.
- Carbone, I., Lazzarotto, T., Ianni, M., Porcellini, E., Forti, P., Masliah, E., Gabrielli, L., and Licastro, F. (2014). Herpes virus in Alzheimer's disease: relation to progression of the disease. *Neurobiol. Aging* *35*, 122–129.
- Cermelli, C., Berti, R., Soldan, S.S., Mayne, M., D'ambrosia, J.M., Ludwin, S.K., and Jacobson, S. (2003). High frequency of human herpesvirus 6 DNA in multiple sclerosis plaques isolated by laser microdissection. *J. Infect. Dis.* *187*, 1377–1387.

- Chan, P.K., Ng, H.K., Hui, M., and Cheng, A.F. (2001). Prevalence and distribution of human herpesvirus 6 variants A and B in adult human brain. *J. Med. Virol.* *64*, 42–46.
- Chapenko, S., Roga, S., Skuja, S., Rasa, S., Cistjakovs, M., Svirskis, S., Zaserska, Z., Groma, V., and Murovska, M. (2016). Detection frequency of human herpesviruses-6A, -6B, and -7 genomic sequences in central nervous system DNA samples from post-mortem individuals with unspecified encephalopathy. *J. Neurovirol.* *22*, 488–497.
- Eimer, W.A., Vijaya Kumar, D.K., Navalpur Shanmugam, N.K., Rodriguez, A.S., Mitchell, T., Washicosky, K.J., György, B., Breakefield, X.O., Tanzi, R.E., and Moir, R.D. (2018). Alzheimer's Disease-Associated β -Amyloid Is Rapidly Seeded by Herpesviridae to Protect against Brain Infection. *Neuron* *100*, 1527–1532.
- Fessel, J. (2018). Amyloid is essential but insufficient for Alzheimer causation: addition of subcellular cofactors is required for dementia. *Int. J. Geriatr. Psychiatry* *33*, e14–e21.
- Flamand, L. (2018). Chromosomal Integration by Human Herpesviruses 6A and 6B. *Adv. Exp. Med. Biol.* *1045*, 209–226.
- Goldschmidt-Clermont, P.J., Volinsky, F.G., LaRosa, S.P., Gilbert, J.R., and Perićak-Vance, M.A. (2019). Time for Well-Powered Controlled Prospective Studies to test a Causal Role for Herpes Viruses in Alzheimer Using Anti-Herpes Drugs. *J. Gerontol. A Biol. Sci. Med. Sci.* glz150.
- Haas, J.G., and Lathe, R. (2018). Microbes and Alzheimer's Disease: New Findings Call for a Paradigm Change. *Trends Neurosci.* *41*, 570–573.
- Hemling, N., Rötttä, M., Rinne, J., Pöllänen, P., Broberg, E., Tapio, V., Vahlberg, T., and Hukkanen, V. (2003). Herpesviruses in brains in Alzheimer's and Parkinson's diseases. *Ann. Neurol.* *54*, 267–271.
- Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A.J., Bright, I.J., Lucero, M.Y., Hiddessen, A.L., Legler, T.C., et al. (2011). High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal. Chem.* *83*, 8604–8610.
- Holmes, C. (2013). Review: systemic inflammation and Alzheimer's disease. *Neuropathol. Appl. Neurobiol.* *39*, 51–68.
- Itzhaki, R.F., Lin, W.R., Shang, D., Wilcock, G.K., Faragher, B., and Jamieson, G.A. (1997). Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* *349*, 241–244.
- Johnson, T.P., Larman, H.B., Lee, M.H., Whitehead, S.S., Kowalak, J., Toro, C., Lau, C.C., Kim, J., Johnson, K.R., Reoma, L.B., et al. (2019). Chronic Dengue Virus Panencephalitis in a Patient with Progressive Dementia with Extrapyramidal Features. *Ann. Neurol.* *86*, 695–703.
- Kumar, D.K., Choi, S.H., Washicosky, K.J., Eimer, W.A., Tucker, S., Ghofrani, J., Lefkowitz, A., McColl, G., Goldstein, L.E., Tanzi, R.E., and Moir, R.D. (2016). Amyloid- β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* *8*, 340ra72.
- Leibovitch, E.C., Brunetto, G.S., Caruso, B., Fenton, K., Ohayon, J., Reich, D.S., and Jacobson, S. (2014). Coinfection of human herpesviruses 6A (HHV-6A) and HHV-6B as demonstrated by novel digital droplet PCR assay. *PLoS ONE* *9*, e92328.
- Leibovitch, E.C., and Jacobson, S. (2018). Viruses in chronic progressive neurologic disease. *Mult. Scler.* *24*, 48–52.
- Leibovitch, E.C., Lin, C.M., Billioux, B.J., Graves, J., Waubant, E., and Jacobson, S. (2019). Prevalence of salivary human herpesviruses in pediatric multiple sclerosis cases and controls. *Mult. Scler.* *25*, 644–652.
- Lin, C.T., Leibovitch, E.C., Almira-Suarez, M.I., and Jacobson, S. (2016). Human herpesvirus multiplex ddPCR detection in brain tissue from low- and high-grade astrocytoma cases and controls. *Infect. Agent. Cancer* *11*, 32.
- Lin, W.R., Wozniak, M.A., Cooper, R.J., Wilcock, G.K., and Itzhaki, R.F. (2002). Herpesviruses in brain and Alzheimer's disease. *J. Pathol.* *197*, 395–402.
- Liu, C., Chyr, J., Zhao, W., Xu, Y., Ji, Z., Tan, H., Soto, C., and Zhou, X.; Alzheimer's Disease Neuroimaging Initiative (2018). Genome-Wide Association and Mechanistic Studies Indicate That Immune Response Contributes to Alzheimer's Disease Development. *Front. Genet.* *9*, 410.
- Lövheim, H., Olsson, J., Weidung, B., Johansson, A., Eriksson, S., Hallmans, G., and Elgh, F. (2018). Interaction between Cytomegalovirus and Herpes Simplex Virus Type 1 Associated with the Risk of Alzheimer's Disease Development. *J. Alzheimers Dis.* *67*, 939–945.
- Miklossy, J. (2015). Historic evidence to support a causal relationship between spirochetal infections and Alzheimer's disease. *Front. Aging Neurosci.* *7*, 46.
- Moir, R.D., and Tanzi, R.E. (2019). Low Evolutionary Selection Pressure in Senescence Does Not Explain the Persistence of A β in the Vertebrate Genome. *Front. Aging Neurosci.* *11*, 70.
- Nath, A. (2015). Neurologic Complications of Human Immunodeficiency Virus Infection. *Continuum (Minneapolis, Minn.)* *21* (6 Neuroinfectious Disease), 1557–1576.
- Piacentini, R., De Chiara, G., Li Puma, D.D., Ripoli, C., Marcocci, M.E., Garaci, E., Palamara, A.T., and Grassi, C. (2014). HSV-1 and Alzheimer's disease: more than a hypothesis. *Front. Pharmacol.* *5*, 97.
- Qin, Q., and Li, Y. (2019). Herpesviral infections and antimicrobial protection for Alzheimer's disease: Implications for prevention and treatment. *J. Med. Virol.* *91*, 1368–1377.
- Readhead, B., Haure-Mirande, J.V., Funk, C.C., Richards, M.A., Shannon, P., Haroutunian, V., Sano, M., Liang, W.S., Beckmann, N.D., Price, N.D., et al. (2018). Multiscale Analysis of Independent Alzheimer's Cohorts Finds Disruption of Molecular, Genetic, and Clinical Networks by Human Herpesvirus. *Neuron* *99*, 64–82 e7.
- Regen, F., Hellmann-Regen, J., Costantini, E., and Reale, M. (2017). Neuroinflammation and Alzheimer's Disease: Implications for Microglial Activation. *Curr. Alzheimer Res.* *14*, 1140–1148.
- Reoma, L.B., Trindade, C.J., Monaco, M.C., Solis, J., Montojo, M.G., Vu, P., Johnson, K., Beck, E., Nair, G., Khan, O.I., et al. (2019). Fetal encephalopathy with wild-type JC virus and ruxolitinib therapy. *Ann. Neurol.* *86*, 878–884.
- Rizzo, R., Bortolotti, D., Gentili, V., Rotola, A., Bolzani, S., Caselli, E., Tola, M.R., and Di Luca, D. (2019). KIR2DS2/KIR2DL2/HLA-C1 Haplotype Is Associated with Alzheimer's Disease: Implication for the Role of Herpesvirus Infections. *J. Alzheimers Dis.* *67*, 1379–1389.
- Romeo, M.A., Faggioni, A., and Cirone, M. (2019a). Could autophagy dysregulation link neurotropic viruses to Alzheimer's disease? *Neural Regen. Res.* *14*, 1503–1506.
- Romeo, M.A., Masuelli, L., Gaeta, A., Nazzari, C., Granato, M., Gilardini Montani, M.S., Faggioni, A., and Cirone, M. (2019b). Impact of HHV-6A and HHV-6B lytic infection on autophagy and endoplasmic reticulum stress. *J. Gen. Virol.* *100*, 89–98.
- Soscia, S.J., Kirby, J.E., Washicosky, K.J., Tucker, S.M., Ingelsson, M., Hyman, B., Burton, M.A., Goldstein, L.E., Duong, S., Tanzi, R.E., and Moir, R.D. (2010). The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. *PLoS ONE* *5*, e9505.
- Spitzer, P., Condic, M., Herrmann, M., Oberstein, T.J., Scharin-Mehlmann, M., Gilbert, D.F., Friedrich, O., Grömer, T., Kornhuber, J., Lang, R., and Maler, J.M. (2016). Amyloidogenic amyloid- β -peptide variants induce microbial agglutination and exert antimicrobial activity. *Sci. Rep.* *6*, 32228.
- Talwar, P., Gupta, R., Kushwaha, S., Agarwal, R., Saso, L., Kukreti, S., and Kukreti, R. (2019). Viral Induced Oxidative and Inflammatory Response in Alzheimer's Disease Pathogenesis with Identification of Potential Drug Candidates: A Systematic Review using Systems Biology Approach. *Curr. Neuropharmacol.* *17*, 352–365.
- Virtanen, J.O., Wohler, J., Fenton, K., Reich, D.S., and Jacobson, S. (2014). Oligoclonal bands in multiple sclerosis reactive against two herpesviruses and association with magnetic resonance imaging findings. *Mult. Scler.* *20*, 27–34.
- Walker, M.A., Peadarallu, C.S., Ojesina, A.I., Bullman, S., Sharpe, T., Whelan, C.W., and Meyerson, M. (2018). GATK PathSeq: a customizable computational tool for the discovery and identification of microbial sequences in libraries from eukaryotic hosts. *Bioinformatics* *34*, 4287–4289.

- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Rowan, M.J., and Selkoe, D.J. (2002). Amyloid-beta oligomers: their production, toxicity and therapeutic inhibition. *Biochem. Soc. Trans.* 30, 552–557.
- Wang, M., Beckmann, N.D., Roussos, P., Wang, E., Zhou, X., Wang, Q., Ming, C., Neff, R., Ma, W., Fullard, J.F., et al. (2018). The Mount Sinai cohort of large-scale genomic, transcriptomic and proteomic data in Alzheimer's disease. *Sci. Data* 5, 180185.
- White, M.R., Kandel, R., Tripathi, S., Condon, D., Qi, L., Taubenberger, J., and Hartshorn, K.L. (2014). Alzheimer's associated β -amyloid protein inhibits influenza A virus and modulates viral interactions with phagocytes. *PLoS ONE* 9, e101364.
- Wipfler, P., Dunn, N., Beiki, O., Trinkka, E., and Fogdell-Hahn, A. (2018). The Viral Hypothesis of Mesial Temporal Lobe Epilepsy - Is Human Herpes Virus-6 the Missing Link? A systematic review and meta-analysis. *Seizure* 54, 33–40.
- Wozniak, M.A., Mee, A.P., and Itzhaki, R.F. (2009). Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. *J. Pathol.* 217, 131–138.
- Yao, K., Honarmand, S., Espinosa, A., Akhyani, N., Glaser, C., and Jacobson, S. (2009). Detection of human herpesvirus-6 in cerebrospinal fluid of patients with encephalitis. *Ann. Neurol.* 65, 257–267.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
MSBB RNA-seq	https://www.synapse.org/#!/Synapse:syn20801188	syn20801188
ROSMAP RNA-seq	https://www.synapse.org/#!/Synapse:syn3388564	syn3388564
PathSeq Analysis of MSBB and ROSMAP Data	This paper	https://doi.org/10.17632/y6t6rvsd6m.2
Oligonucleotides		
HHV-6A U57 Probe: FAM-CTGGAAGTGTATAATAGG-MGBNFQ	Leibovitch et al., 2014	N/A
HHV-6B U57 Probe: VIC-CTGGAGCTGTACAACAG - MGBNFQ	Leibovitch et al., 2014	N/A
HHV-6AB U57 Forward Primer: CCGTGGGATCGTCTAAAATTATAGATGT	Leibovitch et al., 2014	N/A
HHV-6AB Reverse Primer: CCACTAGTCCGGACGGATAA	Leibovitch et al., 2014	N/A
RPP30 Probe: VIC-CTGACCTGAAGGCTCT-MGBNFQ	Hindson et al., 2011	N/A
RPP30 Forward Primer: GATTGGA CCTGCGAGCG	Hindson et al., 2011	N/A
RPP30 Reverse Primer: GCGGCTGT CTCCACAAGT	Hindson et al., 2011	N/A
Software and Algorithms		
R	https://cran.r-project.org/	N/A
gatK	https://software.broadinstitute.org/gatk/download/	RRID:SCR_001876
PathSeqPipelineSpark	https://gatkforums.broadinstitute.org/gatk/discussion/10913/how-to-run-the-pathseq-pipeline	RRID:SCR_005203
samtools	http://samtools.sourceforge.net/	RRID:SCR_002105

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Steven Jacobson (jacobsons@ninds.nih.gov). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

A gender breakdown of all subjects included in each of the datasets included in this study is described in [Table S2](#).

METHOD DETAILS

Screening for Virus Using RNA-Seq Data

The “PathSeq” tool developed by the BROAD Institute was used (<http://software.broadinstitute.org/pathseq/>). Two different RNA-seq datasets available for download from the Synapse database (<https://www.synapse.org/>) were screened. The first dataset consisted of RNA-seq data generated as part of the Mount Sinai Brain Bank (MSBB) study (<https://www.synapse.org/#!/Synapse:syn3159438>). For this study, RNA was collected post-mortem from up to five different regions of the brain (Brodmann Areas 10, 22, 36 and 44) for more than 200 individuals diagnosed with Alzheimer’s Disease (AD) then sequenced (<https://www.synapse.org/#!/Synapse:syn20801188>). The second dataset consisted of RNA-seq data generated as part of the Religious Orders Study and

Memory and Aging Project (ROSMAP). For this study/project, RNA was collected post-mortem from the gray matter of the dorsolateral prefrontal cortex for 605 individuals diagnosed with AD (<https://www.synapse.org/#!Synapse:syn3219045>) then sequenced (<https://www.synapse.org/#!Synapse:syn3388564>). Sequence files downloaded from the MSBB study were the “unmapped” reads in .fastq format. Sequence files downloaded from the ROSMAP study were those in .bam format. Prior to running the “PathSeq” tool on these data, the MSBB files were converted into uBAM format using the “FastqToSam” command supported in the picard tool suite (<https://broadinstitute.github.io/picard/>). While, ROSMAP files were similarly converted using the “view -b -f 4” command supported in the samtools suite (<http://samtools.sourceforge.net/>) followed by use of the “RevertSam” command supported in the picard tool suite. For running the “PathSeq” tool, the “PathSeqPipelineSpark” command supported in the gatk tool suite (<https://software.broadinstitute.org/gatk/download/>) was passed under default parameters (<https://gatkforums.broadinstitute.org/gatk/discussion/10913/how-to-run-the-pathseq-pipeline>) using pre-built references (<https://software.broadinstitute.org/gatk/download/bundle>). The total number of microbes screened for upon running the “PathSeq” tool was 25,917; including 118 human viruses. Detection scores for these viruses per sequence file output from the “PathSeq” tool were then imported into R (<https://cran.r-project.org/>), collated by study/project, visually inspected, and summarized using standard supported commands.

Screening for Virus Using Droplet Digital PCR (ddPCR)

DNA extracted from brain material was collected from two different repositories. HHV-6A and -6B viral load was quantified using droplet digital PCR (ddPCR) as previously described (Leibovitch et al., 2014). Briefly, the HHV-6 primer and probe sequences used (Key Resources Table) were designed based on the reference genomes NC_001664 for HHV-6A, strain U1102, and NC_000898 for HHV-6B, strain Z29. Primers amplify the U57 region encoding the major capsid protein. Ribonuclease P protein subunit P30 (RPP30) was used as a reference gene, as all diploid cells contain two copies of the gene. Each probe was fluorescently labeled, with the HHV-6A probe FAM-MGBNFQ-labeled, while both the HHV-6B and RPP30 probes were VIC-MGBNFQ-labeled. For each DNA sample, the primers and probes were triplexed, with the final concentrations of 900 nM per primer and 250 nM per probe for HHV-6A and HHV-6B, while the final concentrations of RPP30 were 450 nM per primer and 125 nM per probe. DdPCR procedures and analysis of results followed those previously described by Leibovitch et al. (2014). Positive samples were confirmed by an independent re-test.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis of PathSeq Results

Each brain in which one or more brain regions was positive for any of the 6 CNS-related viruses detected (HHV-6A, HHV-6B, CMV, EBV, HIV, or JCV) was counted in a contingency table, and a Chi-square test for independence was performed to determine if there was any relationship between viral detection and AD classification. A separate chi-square analysis was performed for each virus in both MSBB and ROSMAP.

Quantification of ddPCR Results

Fluorescence data for each well were analyzed using QuantaSoft software, version 1.7.4.0917 (Bio-Rad, Hercules, CA). Droplet positivity was determined by fluorescence intensity; only droplets above a manually determined minimum amplitude threshold were counted as positive. For a given sample, target copies per μl were calculated by averaging over all replicate wells, and cellular DNA input was calculated by halving the number of RPP30 copies, as there are two copies of RPP30 per diploid cell. PBMC data are represented as viral copies per 10^6 cells.

Statistical Analysis of ddPCR Results

In both JHBRC and ROSMAP, each non-AD control sample set was compared to AD samples using an unpaired t test of the viral loads of all samples tested. In addition to this analysis of the magnitude of viral load, the frequency of HHV-6 detection was compared between AD and non-AD controls using a chi-square test.

DATA AND CODE AVAILABILITY

PathSeq code is available from the Broad Institute at: <http://software.broadinstitute.org/pathseq/>. RNA-seq datasets from MSBB and ROSMAP are available for download at <https://www.synapse.org/#!Synapse:syn3159438> (MSBB) and <https://www.synapse.org/#!Synapse:syn3388564> (ROSMAP). All PathSeq results from this study have been captured in a single excel workbook available at Mendeley Data (<https://doi.org/10.17632/y6t6rvsd6m.2>).