Human Herpes Virus 6 (HHV-6) - Pathogen or Passenger? A pilot study of clinical laboratory data and next generation sequencing

Colin Sharp[a,b], Tanya Golubchik[c,d], William F. Gregory[a], Anna L. McNaughton[e], Nicholas Gow[f], Mathyruban Selvaratnam[f], Alina Mirea[f], Dona Foster[g], Monique Andersson[f], Paul Klenerman[e,f], Katie Jeffery[f], Philippa C. Matthews[e,f#]

[a] The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, Edinburgh, Scotland, UK

[b] Edinburgh Genomics, Ashworth Laboratories, University of Edinburgh, Edinburgh, EH9 3FL, Scotland, UK

[c] The Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford, OX3 7BN, UK

[d] Big Data Institute, University of Oxford, Old Road, Oxford, OX3 7FZ, UK

[e] Nuffield Department of Medicine, Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, Oxford, OX1 3SY, UK

[f] Department of Infectious Diseases and Microbiology, Oxford University Hospitals NHS Foundation Trust, John Radcliffe Hospital, Headley Way, Headington, Oxford, OX3 9DU, UK

[g] NIHR Biomedical Research Centre, Nuffield Department of Medicine, University of Oxford, John Radcliffe Hospital, Headley Way, Headington, Oxford, OX3 9DU, UK

Corresponding author: Philippa Matthews;

Email: philippa.matthews@ndm.ox.ac.uk

KEY WORDS: Human herpesvirus 6, diagnosis, encephalitis, haematology, metagenomics, next generation sequencing

RUNNING TITLE: HHV-6 epidemiology and diagnostics in a UK cohort

ABSTRACT

Background: Human herpes virus 6 (HHV-6) is a ubiquitous organism that can cause a variety of clinical syndromes ranging from short-lived rash and fever through to life-threatening encephalitis.

Objectives: We set out to generate observational data regarding the epidemiology of HHV-6 infection in clinical samples from a UK teaching hospital and to compare different diagnostic approaches.

Study design: First, we scrutinized HHV-6 detection in samples submitted to our hospital laboratory through routine diagnostic pathways. Second, we undertook a pilot study using Illumina next generation sequencing (NGS) to determine the frequency of HHV-6 in CSF and respiratory samples that were initially submitted to the laboratory for other diagnostic tests.

Results: Of 72 samples tested for HHV-6 by PCR at the request of a clinician, 24 (33%) were positive for HHV-6. The majority of these patients were under the care of the haematology team (30/41, 73%), and there was a borderline association between HHV-6 detection and both Graft versus Host Disease (GvHD) and Central nervous system (CNS) disease (p=0.05 in each case). We confirmed detection of HHV-6 DNA using NGS in 4/20 (20%) CSF and respiratory samples.

Conclusions: HHV-6 is common in clinical samples submitted from a high-risk haematology population, and enhanced screening of this group should be considered. NGS can be used to identify HHV-6 from a complex microbiomee, but further controls are required to define the sensitivity and specificity, and to correlate these results with clinical disease. Our results underpin ongoing efforts to develop NGS technology for viral diagnostics.

ABBREVIATIONS

- cDNA – complementary DNA
- CiHHV-6 – Chromosomally integrated HHV-6
- CMV – Cytomegalovirus
- CNS – central nervous system
- CSF – cerebrospinal fluid
- EPR – Electronic patient record
- GvHD – graft versus host disease
- HHV-6 - Human herpes-virus-6
- HLA – human leucocyte antigen
- HSCT - Haematopoietic stem cell transplant
- HSV – Herpes Simplex Virus
- HTLV – Human T cell lymphotrophic virus
- NGS – Next Generation Sequencing
- OUH – Oxford University Hospitals NHS Foundation Trust
- PCR – Polymerase Chain Reaction
- RSV – Respiratory Syncytial Virus
- VZV – Varicella Zoster Virus

BACKGROUND

Human herpes-virus-6 (HHV-6) is a human beta-herpesvirus (1). Like its close relative human cytomegalovirus (CMV), it is ubiquitous, has the potential for latency followed by chronic low level replication or reactivation, and may modulate immune responses to other pathogens (2-5). In children, is usually asymptomatic or associated with self-limiting fever and rash. It is also associated with encephalitis either as a primary agent (6) or as a result of reactivation in the setting of encephalitis / meningitis caused by other pathogens, in which context it appears generally benign (7). At the other end of the spectrum, HHV-6 can reactivate in the context of severe sepsis (8) and is a cause of potentially life-threatening pathology in patients with haematological malignancy, usually following HSCT (haematopoietic stem cell transplant) (9-15).

HHV-6 variants A and B share approximately 90% homology (16). HHV-6A accounts for more CiHHV-6 (17), while HHV-6B is associated with acute infection, childhood rash/fever (4), and reactivation following HSCT (16). Although HHV-6A is less common in CNS disease (18), it may be more aggressive when present (16). HHV-6 transmission is predominantly via respiratory secretions or saliva, but can also be vertical as a result of chromosomally-integrated HHV-6, 'CiHHV-6' (17, 19), leading either to episodic reactivation (17), or to persistent viraemia (typically ≥5.5 log10 copies/ml (~300,000 copies/ml) in blood (20)).

HHV-6 laboratory diagnostics raise a number of challenges: what sample type to test, in which patient groups to focus, and how to interpret a positive test result. There is increasing interest in 'next generation sequencing' (NGS) approaches to diagnosis of many pathogens (21-25) but optimization is required for these pipelines, including tackling high proportions of human reads, differentiating between pathogenic organisms and commensal / environmental flora, and determining thresholds at which the identification of an organism is likely to be clinically significant (26).

OBJECTIVES

We set out to review HHV-6 data from the local diagnostic microbiology laboratory, to determine the patterns of clinical testing for HHV-6. Second, we screened randomly selected CSF and respiratory samples using PCR and NGS in a small pilot study to ascertain the extent to which this virus can be detected in routine samples. Together, these aim to describe the distribution of HHV-6 in local clinical samples, and to evaluate the contribution made by different diagnostic tools, thereby informing ongoing development of laboratory protocols for diagnosis.

STUDY DESIGN

Study site, cohorts and ethics

Clinical data and samples from between 2013-2016 were collected from the microbiology department at Oxford University Hospitals (OUH) NHS Foundation Trust, a large tertiary referral teaching centre in the UK

(http://www.ouh.nhs.uk/). This study pertains to the analysis of two separate sample cohorts (Suppl data set 1):

i. Samples submitted to the laboratory by clinicians with a request for **HHV-6** screening (ID numbers prefixed **HHV**). This is undertaken at the request of the clinical team when deemed clinically relevant;

ii. Samples which had no request for HHV-6 testing, but had completed routine diagnostic laboratory testing for other indications and were used for **v**iral **s**equencing studies (ID numbers prefixed **VS**).

Approval for retrospective collection of clinical and laboratory data was granted by the OUH Clinical Audit Committee (HHV cohort). Testing of consecutive anonymised laboratory samples (VS cohort) was approved by local Research Services and through review via the UK Integrated Research Application System (REC Reference 14/LO/1077).

Collection of local laboratory data (HHV cohort)

We undertook an electronic search of the OUH Microbiology laboratory system to identify all instances of an HHV-6 test (antibody or viral load) being requested over three years commencing 1-Jan-2013, and recorded age, sex, sample type and patient location. We used the Electronic Patient Record (EPR) to determine underlying diagnosis. Follow-up data for survivors were available for a median of 25 months (range 316 – 1374 days). Diagnostic tests were undertaken by in-house real time PCR assays (27) at two different National Reference Laboratories (Colindale up to 1-Oct-2014, and subsequently Bristol).

155

156 ***Data collection and statistical analysis***

157 We used GraphPad Prism v.6.0f for statistical analysis with Fisher's Exact
158 Test to identify differences between binary groups, and the Mann Whitney U
159 test for continuous variables. Multivariate regression analysis was undertaken
160 using open access on-line software
161 (https://docs.google.com/spreadsheets/u/0/).

162

163 ***Testing CSF and respiratory samples (VS cohort)***

164 We identified 100 CSF samples and 100 respiratory samples (throat swabs
165 (n=22), nasopharyngeal aspirates (n=42), endotracheal aspirates (n=4), and
166 bronchoalveolar lavage samples (n=32)) representing a 'high risk' subgroup
167 based on the following criteria:

168 i. For CSF, the clinical request for testing included viral causes of
169 meningitis and encephalitis;
170 ii. For respiratory samples, the patient had a clinical history of
171 immunocompromise and/or was on the intensive care unit.

172

173 ***PCR and NGS (VS cohort)***

174 Samples had all undergone clinical laboratory testing and were stored at -
175 80°C prior to further processing. We selected consecutive samples; the only
176 exclusion criterion was inadequate sample volume (<200 ul). For each
177 sample, we documented patient age group and clinical location, and recorded
178 the clinical information supplied with the sample and routine microbiology
179 laboratory data.

Nucleic acids were extracted from 200µl of each sample using the AllPrep DNA/RNA Mini Kit (Qiagen) and recovered in 30µl of nuclease-free water. To allow for broad detection of HHV-6 and other known herpesviruses, 4ul of DNA was used as template for a consensus PCR primer as previously published (28). The results were visualised on 2% agarose gels and amplicons from positive reactions were cut from the gels for sequencing. Direct amplicon sequencing was performed using BigDye Terminator v3.1 (Applied Biosystems) according to the manufacturer's instructions with both second round primers. Sequencing reactions were read by Edinburgh Genomics and assembled using SSE v1.2 (29).

We selected a random subset of 20 samples for Illumina sequencing. Methods are described in detail in another manuscript (30).

RESULTS

Routine clinical samples received for HHV-6 testing (HHV cohort)

During the three-year study period, our clinical laboratory received 85 samples for HHV-6 testing (Fig 1A; suppl data set 1). In total, 41 patients were tested; 22 M:19 F; median age 52 years (range 2-71; IQR 39-61). Central nervous system (CNS) disease (encephalitis, encephalopathy, meningism, seizures) was present in 9/41 (22%). Most patients were under haematology care (30/41; 73%), of these, 5/30 (17%) had Graft versus Host Disease (GvHD). 72/85 samples were tested for HHV-6 DNA by PCR at the reference laboratory; 24/72 (33%) were positive. Among individual patients tested, 15/41

(37%) were PCR positive at ≥1 timepoint (Fig 1A). Of seven samples that were subtyped, all were HHV-6B.

Positive HHV-6 status was not statistically associated with age, sex, or haematological malignancy (Table 1). There was a borderline association with both GvHD and CNS disease (both p=0.05; Table 1; Fig. 2A and B), but this is difficult to interpret as patients in these groups are more likely to be selected for HHV6 testing. On multiple logistic regression analysis, there was no relationship between HHV-6-positivity and any other characteristic (Table 1).

In this cohort, 23/41 (56%) of patients died, at a median age of 56 years. Among these, 11/23 (48%) had tested HHV-6-PCR positive, compared to 4/18 (22%) of surviving patients (p=0.1). All those who died did so within 19 months of the HHV-6 test (range 1-552 days, median 163 days), and all patients with HHV6 detected in the CSF died (Fig 2C). We did not have sufficient clinical data to determine cause of death, and HHV6 may be a bystander in this complex cohort.

Quantification of HHV-6 viral load in blood and CSF (HHV cohort)

HHV-6 DNA was quantified in 23 samples from ten patients (Fig 2C). The levels varied from below the threshold for accurate quantification, to patient HHV-012 with $>1.0x10^6$ copies/ml in blood and $2.0x10^8$ DNA copies/ml in CSF, suggesting CiHHV-6. Patient HHV-007, an adult with haematological malignancy, had HHV-6 DNA detected in both blood and CSF ($9x10^4$ DNA copies/ml vs. $3x10^5$ copies/ml, respectively). This patient had limbic

encephalitis, and the raised HHV-6 titre in CSF compared to blood is in keeping with a localized CNS pathology caused by the virus.

Among a total of 72 tests, 46 were longitudinal samples from thirteen individual patients. There was no statistical association between multiple HHV-6 tests and other factors that might predict disease severity ($p>0.1$ for age, sex, mortality, haematological malignancy, transplant, intensive care location, documented co-infection in multiple regression analysis).

Screening laboratory samples for HHV-6 by PCR (VS cohort)

We screened CSF (n=100) and respiratory (n=100) samples for herpesvirus DNA by PCR and sequencing (Fig 1B; Suppl data set 1), identifying HHV-6 in 3/100 CSF samples and 5/100 respiratory samples (Table 2). Four of the eight positive cases were age <5 years, representing the age group in whom primary HHV-6 infection is most likely. However, in four samples (two adults and two children) an alternative pathogen was identified by the clinical lab (Table 2), illustrating the difficulty in distinguishing between HHV-6 as a primary pathogen, a co-infecting agent contributing to pathology, or an innocent bystander.

The overall HHV-6 prevalence of 4% in this random group of samples is significantly lower than the 33% rate obtained from the samples in which the clinician had requested HHV-6 testing ($p<0.0001$; Fisher's Exact Test). This suggests that the targeting of the highest risk groups (primarily patients with haematological malignancy) for HHV-6 testing is appropriate, and that the

high prevalence of detectable HHV-6 DNA in this group is not merely reflective of universal reactivation of herpesviruses in a hospital cohort.

Screening laboratory samples for HHV-6 DNA by NGS (VS cohort)

We screened a subset of 20 samples (10 CSF and 10 respiratory; Fig 1B) using a metagenomic (NGS) approach (30), identifying HHV-6 in four samples that we deemed 'positive'; one CSF and three respiratory samples (Table 3; Fig 3). The number and distribution of reads in each sample is shown in Fig 4A. Sample VS183 was designated HHV-6B by Kraken, while the other three positive samples were identified as HHV-6A (Fig 4B). The coverage of the HHV-6 genome was incomplete, but multiple reads distributed across the genome (Fig 4B), add confidence to the conclusion that HHV-6 DNA is genuinely present in these samples. The sequence data have been uploaded to European Nucleotide Archive (ENA) https://www.ebi.ac.uk/ena; HHV6 sequence accession numbers ERS1980462 (sample ID VS067), ERS1980463 (VS183), ERS1980464 (VS200) and ERS1980465 (VS207). Links to the full metagenomic sequence set can be found in our supporting Data Note (30).

In VS067, the clinical syndrome was not explained by other diagnostic results, and HHV-6 infection was a plausible agent of the clinical syndrome (meningoencephalitis). In the three other cases, other primary pathogens had been identified (Table 3), although it is plausible that HHV-6 could have been a contributory agent.

Among the total of eight samples that were HHV-6 positive by conventional PCR, three were tested on the NGS pipeline (samples VS114, VS121 and VS183). HHV-6 reads were detected by NGS in all three, although two had low numbers of reads leading us to classify them as equivocal (Fig 4A,B). Conversely, in four samples where HHV-6 was detected by NGS, only one of these was positive by PCR.

DISCUSSION

This preliminary study provides insight into the distribution of HHV-6 in a range of samples in a UK teaching hospital, with the long-term aim of informing improvements in diagnostic testing. Although our NGS data only represent a small pilot study, we have identified only a single other reference to date that describes a metagenomic approach to the diagnosis of HHV-6 infection (6). Although HHV-6 can reactivate in the context of any critical illness (8), we here found a higher prevalence in clinical samples taken from a high-risk group (HHV cohort) than in laboratory samples representing an unwell hospital population (VS cohort), suggesting that HHV-6 is not simply reactivating across the board in hospitalized patients. The combination of a high rate of HHV-6 DNA detection and the high mortality in the clinical (HHV) cohort, suggest that we should consider lowering our threshold for testing in this context. Longitudinal HHV-6 PCR testing should generally be reserved for monitoring response to therapy, but if a high index of suspicion exists (e.g. a profoundly immunosuppressed patient who becomes encephalopathic in the absence of an alternative explanation), then serial testing may be helpful.

This study was not designed to evaluate the use or outcome of antiviral therapy as this information could not robustly be captured retrospectively. There are some data to suggest good outcomes from treatment of symptomatic viraemia (31), with ganciclovir and/or foscarnet. However, there is no universally agreed definition of clinical disease or threshold for therapy and this area is not well informed by clinical trials (32); the gains made by testing early have to be carefully balanced against the possibility of identifying patients with bystander viral reactivation in whom the toxicity and side-effects of treatment would not be justified. Further studies, including NGS, may determine whether different clinical syndromes are consistently associated with the two HHV-6 subtypes.

For samples with low HHV-6 copy numbers, PCR is anticipated to be more sensitive than NGS; this is illustrated by two samples that tested positive by PCR but were equivocal by NGS. However, the overall proportion of samples testing positive for HHV-6 DNA was higher by NGS than by PCR. This may at least in part be accounted for by PCR using highly degenerate herpesvirus primers, followed by sequencing primers that have a higher degree of sequence homology for HHV-6B than HHV-6A (28).

In screening by NGS, we identified HHV-6 in 4/20 (20%) samples, using a combination of read numbers and genome coverage to infer positivity (33). Multiple considerations feed into the interpretation of NGS data (Fig 5). The four patients positive for HHV-6 by NGS in this cohort all had clinical syndromes that could be compatible with HHV-6 infection, but due to a limited

dataset we cannot attribute causality. Interestingly, positive HHV-6 PCR from young children with severe respiratory tract infections suggest a potential pathological role of the virus that is not well described in this population to date. In future, in situations when HHV-6 detection is deemed significant, this could support the introduction of antiviral therapy, and/or reduce exposure to broad-spectrum antibiotics.

The number of clinical requests for HHV-6 testing each year is small, even in a tertiary referral teaching hospital. By nature, our clinical sample set (HHV cohort) was strongly skewed towards sampling a high-risk population. Our data quality is dependent upon the completeness of electronic data, and it was not possible to determine temporality. For ongoing NGS work, priorities are screening blood samples, and development of positive internal controls to determining sensitivity. An international control standard for HHV-6 is currently being prepared by the National Institute for Biological Standards and Control (NIBSC).

Although it is doubtless at times a benign passenger, HHV-6 is indeed significantly associated with the clinical syndromes that arise in patients with profound immunocompromise in the haematology setting, and may have a role in other syndromes, including respiratory infections in children. Metagenomic approaches to clinical diagnostics are accelerating, and as additional data become available, increasing insights will be gained into the interpretation of these results.

355

356 **FUNDING**

357 PCM is funded by a Wellcome Trust Intermediate Fellowship, grant number
358 110110 and received salary from the NIHR during the clinical data collection
359 phase of this work. A project grant to PCM from The British Infection
360 Association (BIA; https://www.britishinfection.org/) covered the costs of
361 laboratory staff and consumables for undertaking PCR and metagenomic
362 screening of CSF and respiratory samples. The funders had no role in study
363 design, data collection and interpretation, or the decision to submit the work
364 for publication.

365

366 **ACKNOWLEDGEMENTS**

367 We are grateful to the Centre for Genomic Research (CGR), University of
368 Liverpool, UK for running the HiSeq platform. Thank you to Dr Peter Muir
369 (Public Health England South West Regional Laboratory, Southmead
370 Hospital, Bristol), who provided valuable advice and discussion during data
371 collection. A subset of these work were presented at the UK Federation of
372 Infection Meeting, 2017 (34).

373

374 **CONFLICTS OF INTEREST**

375 None to declare.

376

377

AUTHORSHIP

Conceived and designed the experiments: CS, PK, PCM; Applied for ethics permission: PCM; Collected and curated clinical samples and data: MS, AM, NG, MA, KJ; Undertook laboratory work: CS, WFG; Analysed and presented the data: CS, TG, ALM, PCM; Wrote the article: CS, TG, ALM, DF, PCM, with feedback from all co-authors; Approved the final article: all authors.

FIGURE LEGENDS

Figure 1: Schematic summary of work flow and output of HHV-6 testing among clinical samples from a UK teaching hospital. A: Flow diagram showing number of samples submitted to a hospital diagnostic microbiology laboratory with a clinical request for HHV-6 testing (sample ID's prefixed with HHV). *'Wrong test' indicates request for HHV-6 antibody (rather than PCR). B: Flow diagram showing consecutive random samples (sample ID's prefixed with VS) screened for herpesviruses (i) by PCR with HHV-6 confirmed by sequencing, and (ii) by a metagenomic approach. These samples had been submitted to the clinical laboratory for other reasons, and had reached the end of their diagnostic testing pathway.

Figure 2: Results of clinical laboratory testing for HHV-6 in blood and CSF samples at the request of the clinical team. A: Relationship between presence or absence of Graft vs Host Disease (GvHD) and HHV-6 status; P value by Fisher's exact test. B: Relationship between presence or absence of a clinical Central Nervous System (CNS) syndrome and HHV-6 status; P value by Fisher's exact test. C: HHV-6 viral loads in blood and CSF; P value

403 by Mann Whitney test; individuals who died are shown in red. In all three
404 panels, the numbers at the top of each column show the total number of
405 patients represented.
406
407 **Figure 3: Multi-layered pie charts generated to visualize the metavirome**
408 **from a respiratory sample taken from a child.** Krona was used to generate
409 the metagenomic visualization of these data (35). The sample was a
410 nasopharyngeal aspirate taken from patient ID VS183 (a child age <5 with a
411 clinical syndrome described by the requesting clinician as 'viral infection'). (A)
412 HHV-6B (in pink) shown as a proportion of all viral reads; (B) HHV-6B (in pink)
413 shown as a proportion of all virus contigs. The other two predominant viruses
414 represented in both panels are Torque Teno Mini Virus (in red), a ubiquitous
415 and non-pathogenic virus, and Enterobacteria phage Phi X (in purple), which
416 is an artefact of the sequencing method (spike used for positive control in
417 Illumina sequencing run); these illustrate a high proportion of sequence reads
418 generated from organisms that are not clinically significant.
419
420 **Figure 4: Detection of HHV-6 DNA in unselected CSF and respiratory**
421 **samples.** A: Read numbers determined by next generation sequencing
422 (NGS) according to sample type. The y-axis refers to the number of uniquely
423 mapping HHV6 reads, representing deduplicated read numbers (Q>30). The
424 areas shaded green, blue and yellow represent suggested thresholds for
425 samples to be classified as negative, equivocal or positive, respectively. White
426 gaps between the colours illustrate that the exact position of the boundaries
427 between these areas are uncertain. Those classified as equivocal were all

428 respiratory samples, and the sequences clustered in the repeat region of the
429 genome, suggesting lower specificity for HHV-6.
430 B: Plots generated with Burrows-Wheeler Aligner (BWA) to illustrate coverage
431 of HHV-6 genomes against consensus. The x-axis of the plot represents the
432 full length genome for HHV-6, with HHV-6A shown in triangles, and HHV-6B
433 shown in circles. From a total of 20 samples tested, we show data for samples
434 from which we generated any HHV-6 reads. The beginning position of each
435 read is indicated. The grey bars indicate repeat regions (low variability) as
436 defined in the methods; for a sample to be considered positive, we stipulated
437 that the reads should fall outside these regions of the genome.
438
439 **Figure 5: Suggested algorithm showing process of determining the**
440 **significance of an organism identified from a clinical sample by next**
441 **generation sequencing (NGS).** This represents a structure that can be
442 applied to bioinformatics and clinical interpretation of metagenomic data. We
443 recognize that the approach and thresholds are different for different
444 organisms, and that robust output also depends also on optimization of *in vitro*
445 sample preparation.
446
447

TABLES

Table 1: Relationship between HHV-6 status and clinical/demographic features in 41 patients with samples submitted for HHV-6 testing to a clinical UK microbiology lab.

	HHV-6 PCR positive[a] (n=15 patients)	HHV-6 PCR negative (n=26 patients)	p-value[b] (univariate analysis by Fisher's Exact test)	p-value[b] (multivariate analysis by logistic regression)
Sample type[c]: blood	9/15 (60%)	15/26 (58%)	1.0	0.7
Sample type[c]: CSF	3/15 (20%)	4/26 (15%)	0.7	0.8
Age ≥50 years	10/15 (67%)	13/26 (50%)	0.3	0.3
Male sex	8/15 (53%)	14/26 (54%)	1.0	0.2
Haematological malignancy present	13/15 (87%)	17/26 (65%)	0.2	0.9
Graft versus host disease present	4/15 (27%)	1/26 (4%)	**0.05**	0.1
CNS[d] disease present	6/15 (40%)	3/26 (12%)	**0.05**	0.1
Solid organ transplant present	0/15 (0%)	2/26 (8%)	0.5	1.0
Death[e]	11/15 (73%)	11/25 (44%)	0.1	0.2

[a] In the case of patients tested more than once, any instance of PCR positive is recorded as positive; [b] Sample types were not documented for 3 patients testing PCR-positive, and for 7 patients testing PCR-negative; [c] CNS=central nervous system; [d] Prospective survival data were available for 40/41 patients.

Table 2: Summary of eight clinical samples (from a total of 200 consecutive samples selected for screening) that tested positive for HHV-6 DNA by PCR

Study ID number	Sample type	Age group (years)	Location	Clinical details	CSF cell count: total WCC (cells/µl)	CSF cell count: lymphs (cells/µl)	CSF cell count: RBC (cells/µl)	Bacterial culture results from routine clinical lab	Molecular diagnostics (PCR) from routine clinical lab
VS013	CSF	20-24	Emergency Department	Headache, ? SAH	346	342	70	No growth	VZV detected
VS014	CSF	<5	Paediatric ICU	? Meningitis	8	6	8	No growth	No virus detected
VS022	CSF	60-64	Medicine	Confusion, temporal attenuation on CT brain	28	26	28	No growth	HSV type 1 detected
VS114	NPA	<5	Paediatric ICU	? Brochiolitis	n/a	n/a	n/a	Not requested	No organism detected
VS121	NPA	<5	Paediatric ICU	LRTI	n/a	n/a	n/a	Not requested	Rhinovirus detected
VS127	BAL	65-69	Chest Unit	Pneumonia	n/a	n/a	n/a	No growth	No organism detected
VS152	BAL	55-59	Chest Unit	? TB	n/a	n/a	n/a	*Streptococcus viridans*	No organism detected
VS183	NPA	<5	Paediatrics	Viral infection	n/a	n/a	n/a	Not requested	Meta-pneumovirus detected

CSF=cerebrospinal fluid, NPA=nasopharyngeal aspirate, BAL=broncho-alveolar lavage, HSV = Herpes Simplex Virus, ICU=intensive care unit, SAH=subarachnoid haemorrhage, CT=computer tomography, LRTI=lower respiratory tract infection, TB=tuberculosis, PCR=polymerase chain reaction, VZV=varicella zoster virus. '?' in clinical details column reflects potential but unconfirmed diagnosis being raised as a query by the requesting clinician.

475 **Table 3: Summary of HHV-6 results from twenty clinical samples screened for**
476 **pathogens by a metagenomic (NGS) approach.**
477

Study ID number	Sample type	Age (years)	Clinical details on request form	CSF Lympho-cyte count (cells/µl)	Clinical laboratory virology result	HHV-6 PCR result	HHV-6 detection by NGS approach
VS011	CSF	<5	Fever	2	No virus detected	Negative	Negative
VS012	CSF	<5	VP shunt	4	No virus detected	Negative	Negative
VS033	CSF	60-64	No data	0	No virus detected	Negative	Negative
VS036	CSF	<5	No data	0	No virus detected	Negative	Negative
VS047	CSF	5-9	No data	0	No virus detected	Negative	Negative
VS060	CSF	40-44	Previous high CSF white cell count (50).	32	No virus detected	Negative	Negative
VS067	CSF	50-54	Confusion, headache, malaise, fever	438	No virus detected	Negative	Positive (BWA HHV-6A, 3653 reads, 1% coverage)
VS071	CSF	70-74	Headache, fever, cognitive impairment	0	No virus detected	Negative	Negative
VS079	CSF	40-44	Sepsis, Confusion, Encephalopathy	2	No virus detected	Negative	Negative
VS088	CSF	20-24	Mixed sensory and motor symptoms	0	No virus detected	Negative	Negative
VS114	Resp (NPA)	75-79	Possible bronchiolitis	N/A	No organism detected	Positive	Equivocal (HHV-6A; BWA mapping within repeat region only)
VS121	Resp (NPA)	<5	Lower respiratory tract infection	N/A	Rhinovirus detected	Positive	Equivocal (HHV-6A; BWA mapping within repeat region only)
VS134	Resp (BAL)	55-59	Neutropenic; sudden respiratory deterioration	N/A	No organism detected	Negative	Equivocal (HHV-6A; BWA mapping within repeat region only)
VS147	Resp (BAL)	50-54	?TB	N/A	No organism detected	Negative	Equivocal (HHV-6A; BWA mapping within repeat region only)
VS167	Resp (NPA)	<5	Tracheostomy, NIV, increasing secretions	N/A	Rhinovirus detected	Negative	Negative
VS177	Resp (NPA)	<5	LRTI, CXR changes	N/A	Meta-pneumo-virus detected	Negative	Negative
VS183	Resp (NPA)	<5	Viral infection	N/A	Meta-pneumo-	Positive	Positive (HHV-6B; 93 reads;

					virus detected		BWA 1% coverage)
VS200	Resp (NPA)	<5	DKA, increased respiratory secretions,	N/A	RSV detected	Negative	Positive (HHV-6A; 7077 reads; BWA 1% coverage)
VS207	Resp (BAL)	<5	Respiratory arrest	N/A	Rhinovirus detected	Negative	Positive (HHV-6A; 11305 reads; BWA 1% coverage)
VS216	Resp (NPA)	<5	LRTI,? TB	N/A	No organism detected	Negative	Equivocal (HHV-6A; BWA mapping within repeat region only)

BAL = broncho-alveolar lavage**;** BWA = Burrows-Wheeler Aligner (33); DKA = diabetic ketoacidosis; LRTI = lower respiratory tract infection; NIV = non invasive ventilation; NGS = next generation sequencing; NPA = naso-pharyngeal aspirate; N/A = not applicable; TB = tuberculosis; VP = ventriculo-peritoneal. Read counts and thresholds for positivity are shown in Fig 4.

SUPPLEMENTARY RESOURCES

Suppl data set 1. Human Herpes Virus 6 (HHV-6): a study of clinical laboratory data and next generation sequencing; https://doi.org/10.6084/m9.figshare.5671153.v1.

This fileset includes the following:

- HHV6 cohort metadata (clinical cohort data to describe patients undergoing clinical testing for HHV-6 infection) as .xlsx and .csv files;
- VS cohort metadata (research cohort data to describe CSF and respiratory samples underdoing screening for HHV-6 infection using PCR and next generation sequencing) as .xlsx and .csv files.

REFERENCES

1. **Salahuddin SZ, Ablashi DV, Markham PD, Josephs SF, Sturzenegger S, Kaplan M, Halligan G, Biberfeld P, Wong-Staal F, Kramarsky B, et al.** 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science **234:**596-601.
2. **De Bolle L, Naesens L, De Clercq E.** 2005. Update on human herpesvirus 6 biology, clinical features, and therapy. Clinical microbiology reviews **18:**217-245.
3. **Adland E, Klenerman P, Goulder P, Matthews PC.** 2015. Ongoing burden of disease and mortality from HIV/CMV coinfection in Africa in the antiretroviral therapy era. Front Microbiol **6:**1016.
4. **Abdel Massih RC, Razonable RR.** 2009. Human herpesvirus 6 infections after liver transplantation. World journal of gastroenterology : WJG **15:**2561-2569.
5. **Loutfy SA, Fawzy M, El-Wakil M, Moneer MM.** 2010. Presence of human herpes virus 6 (HHV6) in pediatric lymphomas: impact on clinical course and association with cytomegalovirus infection. Virol J **7:**287.
6. **Kawada J, Okuno Y, Torii Y, Okada R, Hayano S, Ando S, Kamiya Y, Kojima S, Ito Y.** 2016. Identification of Viruses in Cases of Pediatric Acute Encephalitis and Encephalopathy Using Next-Generation Sequencing. Sci Rep **6:**33452.
7. **Labska K, Roubalova K, Picha D, Maresova V.** 2015. Presence of herpesvirus DNA in cerebrospinal fluid of patients with tick-borne

encephalitis and enteroviral meningoencephalitis. Journal of medical virology **87:**1235-1240.

8. **Ong DSY, Bonten MJM, Spitoni C, Verduyn Lunel FM, Frencken JF, Horn J, Schultz MJ, van der Poll T, Klein Klouwenberg PMC, Cremer OL, Molecular D, Risk Stratification of Sepsis C.** 2017. Epidemiology of Multiple Herpes Viremia in Previously Immunocompetent Patients With Septic Shock. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **64:**1204-1210.

9. **Ogata M, Satou T, Kadota J, Saito N, Yoshida T, Okumura H, Ueki T, Nagafuji K, Kako S, Uoshima N, Tsudo M, Itamura H, Fukuda T.** 2013. Human herpesvirus 6 (HHV-6) reactivation and HHV-6 encephalitis after allogeneic hematopoietic cell transplantation: a multicenter, prospective study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **57:**671-681.

10. **de Pagter PJ, Schuurman R, Keukens L, Schutten M, Cornelissen JJ, van Baarle D, Fries E, Sanders EA, Minnema MC, van der Holt BR, Meijer E, Boelens JJ.** 2013. Human herpes virus 6 reactivation: important predictor for poor outcome after myeloablative, but not non-myeloablative allo-SCT. Bone marrow transplantation **48:**1460-1464.

11. **Schonberger S, Meisel R, Adams O, Pufal Y, Laws HJ, Enczmann J, Dilloo D.** 2010. Prospective, comprehensive, and effective viral monitoring in children undergoing allogeneic hematopoietic stem cell transplantation. Biology of blood and marrow transplantation : journal of

the American Society for Blood and Marrow Transplantation **16:**1428-1435.

12. **Betts BC, Young JA, Ustun C, Cao Q, Weisdorf DJ.** 2011. Human herpesvirus 6 infection after hematopoietic cell transplantation: is routine surveillance necessary? Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation **17:**1562-1568.

13. **Toriumi N, Kobayashi R, Yoshida M, Iguchi A, Sarashina T, Okubo H, Suzuki D, Sano H, Ogata M, Azuma H.** 2014. Risk factors for human herpesvirus 6 reactivation and its relationship with syndrome of inappropriate antidiuretic hormone secretion after stem cell transplantation in pediatric patients. Journal of pediatric hematology/oncology **36:**379-383.

14. **Mori Y, Miyamoto T, Nagafuji K, Kamezaki K, Yamamoto A, Saito N, Kato K, Takenaka K, Iwasaki H, Harada N, Abe Y, Teshima T, Akashi K.** 2010. High incidence of human herpes virus 6-associated encephalitis/myelitis following a second unrelated cord blood transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation **16:**1596-1602.

15. **Ogata M, Oshima K, Ikebe T, Takano K, Kanamori H, Kondo T, Ueda Y, Mori T, Hashimoto H, Ogawa H, Eto T, Ueki T, Miyamoto T, Ichinohe T, Atsuta Y, Fukuda T.** 2017. Clinical characteristics and outcome of human herpesvirus-6 encephalitis after allogeneic

574 hematopoietic stem cell transplantation. Bone marrow transplantation
575 **52:**1563-1570.
576 16. **Ablashi D, Agut H, Alvarez-Lafuente R, Clark DA, Dewhurst S,**
577 **DiLuca D, Flamand L, Frenkel N, Gallo R, Gompels UA, Hollsberg**
578 **P, Jacobson S, Luppi M, Lusso P, Malnati M, Medveczky P, Mori Y,**
579 **Pellett PE, Pritchett JC, Yamanishi K, Yoshikawa T.** 2014.
580 Classification of HHV-6A and HHV-6B as distinct viruses. Arch Virol
581 **159:**863-870.
582 17. **Tweedy J, Spyrou MA, Pearson M, Lassner D, Kuhl U, Gompels**
583 **UA.** 2016. Complete Genome Sequence of Germline Chromosomally
584 Integrated Human Herpesvirus 6A and Analyses Integration Sites
585 Define a New Human Endogenous Virus with Potential to Reactivate
586 as an Emerging Infection. Viruses **8**.
587 18. **Lou J, Wu Y, Cai M, Wu X, Shang S.** 2011. Subtype-specific, probe-
588 based, real-time PCR for detection and typing of human herpesvirus-6
589 encephalitis from pediatric patients under the age of 2 years. Diagn
590 Microbiol Infect Dis **70:**223-229.
591 19. **Hall CB, Caserta MT, Schnabel K, Shelley LM, Marino AS,**
592 **Carnahan JA, Yoo C, Lofthus GK, McDermott MP.** 2008.
593 Chromosomal integration of human herpesvirus 6 is the major mode of
594 congenital human herpesvirus 6 infection. Pediatrics **122:**513-520.
595 20. **Pellett PE, Ablashi DV, Ambros PF, Agut H, Caserta MT,**
596 **Descamps V, Flamand L, Gautheret-Dejean A, Hall CB, Kamble RT,**
597 **Kuehl U, Lassner D, Lautenschlager I, Loomis KS, Luppi M, Lusso**
598 **P, Medveczky PG, Montoya JG, Mori Y, Ogata M, Pritchett JC,**

Rogez S, Seto E, Ward KN, Yoshikawa T, Razonable RR. 2012. Chromosomally integrated human herpesvirus 6: questions and answers. Rev Med Virol **22:**144-155.

21. **Fridholm H, Ostergaard Sorensen L, Rosenstierne MW, Nielsen H, Sellebjerg F, Bengard Andersen A, Fomsgaard A.** 2016. Human pegivirus detected in a patient with severe encephalitis using a metagenomic pan-virus array. J Clin Virol **77:**5-8.

22. **Graf EH, Simmon KE, Tardif KD, Hymas W, Flygare S, Eilbeck K, Yandell M, Schlaberg R.** 2016. Unbiased Detection of Respiratory Viruses by Use of RNA Sequencing-Based Metagenomics: a Systematic Comparison to a Commercial PCR Panel. J Clin Microbiol **54:**1000-1007.

23. **Lecuit M, Eloit M.** 2015. The potential of whole genome NGS for infectious disease diagnosis. Expert Rev Mol Diagn **15:**1517-1519.

24. **Lewandowska DW, Zagordi O, Zbinden A, Schuurmans MM, Schreiber P, Geissberger FD, Huder JB, Boni J, Benden C, Mueller NJ, Trkola A, Huber M.** 2015. Unbiased metagenomic sequencing complements specific routine diagnostic methods and increases chances to detect rare viral strains. Diagn Microbiol Infect Dis **83:**133-138.

25. **Mulcahy-O'Grady H, Workentine ML.** 2016. The Challenge and Potential of Metagenomics in the Clinic. Front Immunol **7:**29.

26. **Bukowska-Osko I, Perlejewski K, Nakamura S, Motooka D, Stokowy T, Kosinska J, Popiel M, Ploski R, Horban A, Lipowski D, Caraballo Cortes K, Pawelczyk A, Demkow U, Stepien A,**

624 **Radkowski M, Laskus T.** 2017. Sensitivity of Next-Generation
625 Sequencing Metagenomic Analysis for Detection of RNA and DNA
626 Viruses in Cerebrospinal Fluid: The Confounding Effect of Background
627 Contamination. Adv Exp Med Biol **944:**53-62.
628 27. **Collot S, Petit B, Bordessoule D, Alain S, Touati M, Denis F,**
629 **Ranger-Rogez S.** 2002. Real-time PCR for quantification of human
630 herpesvirus 6 DNA from lymph nodes and saliva. J Clin Microbiol
631 **40:**2445-2451.
632 28. **VanDevanter DR, Warrener P, Bennett L, Schultz ER, Coulter S,**
633 **Garber RL, Rose TM.** 1996. Detection and analysis of diverse
634 herpesviral species by consensus primer PCR. J Clin Microbiol
635 **34:**1666-1671.
636 29. **Simmonds P.** 2012. SSE: a nucleotide and amino acid sequence
637 analysis platform. BMC Res Notes **5:**50.
638 30. **Sharp C, Golubchik T, Gregory WF, McNaughton AL, Gow N,**
639 **Selvaratnam M, Mirea A, Foster D, Andersson M, Klenerman P,**
640 **Jeffery K, Matthews PC.** 2018. Oxford Screening CSF and
641 Respiratory samples ('OSCAR'): results of a pilot study to screen
642 clinical samples from a diagnostic microbiology laboratory for viruses
643 using Illumina next generation sequencing. BMC Res Notes **11:**120.
644 31. **Colombier MA, Amorim S, Salmona M, Thieblemont C, Legoff J,**
645 **Lafaurie M.** 2017. HHV-6 reactivation as a cause of fever in
646 autologous hematopoietic stem cell transplant recipients. J Infect
647 **75:**155-159.

32. **Ljungman P, de la Camara R, Cordonnier C, Einsele H, Engelhard D, Reusser P, Styczynski J, Ward K, European Conference on Infections in L.** 2008. Management of CMV, HHV-6, HHV-7 and Kaposi-sarcoma herpesvirus (HHV-8) infections in patients with hematological malignancies and after SCT. Bone marrow transplantation **42:**227-240.

33. **Lu J, Breitwieser FP, Thielen P, Salzberg SL.** 2017. Bracken: estimating species abundance in metagenomics data. PeerJ Comput. Sci. **3:e104; DOI 10.7717/peerj-cs.104**.

34. **McNaughton A, Golubchik T, Sharp C, Gregory WF, Selvaratnam M, Mirea A, Klenerman P, Jeffery K, Matthews PC.** 2017. Oxford screening of CSF and Respiratory Samples ('OSCAR'): comparison of routine laboratory diagnostics, multiplex PCR and next generation sequencing for identification of viruses from clinical samples. F1000Research 2017, 6:2130 (poster) (doi: 10.7490/f1000research.1115150.1).

35. **Ondov BD, Bergman NH, Phillippy AM.** 2011. Interactive metagenomic visualization in a Web browser. BMC Bioinformatics **12:**385.

A

85 samples received by clinical lab over 3 years

1 discarded from analysis
(mock sample)

84 test requests for 46 unique patients

12 rejected by lab
- *duplicate; n=5*
- *wrong test; n=5**
- *wrong sample; n=1*
- *insufficient volume; n=1*

N=72 test requests for 41 unique patients

HHV6 DNA positive:
- 24/72 (33%) samples
- 15/41 (37%) patients

B

Consecutive samples identified by clinical microbiology laboratory after completion of diagnostic testing

100 CSF samples

100 respiratory samples

200 samples screened by HHV6 PCR

CSF samples:
3/100 (3%) HHV6 positive

Respiratory samples:
5/100 (5%) HHV6 positive

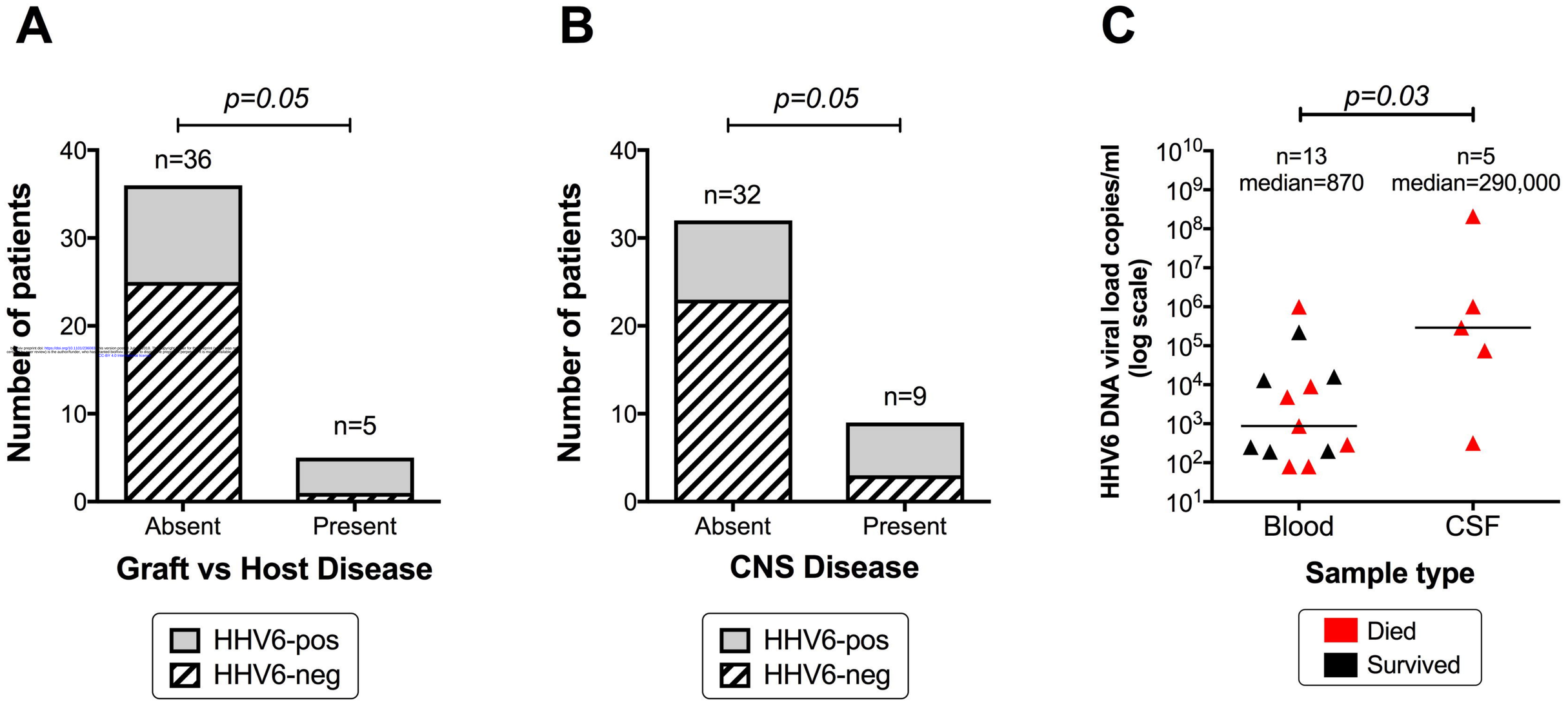
10 CSF samples

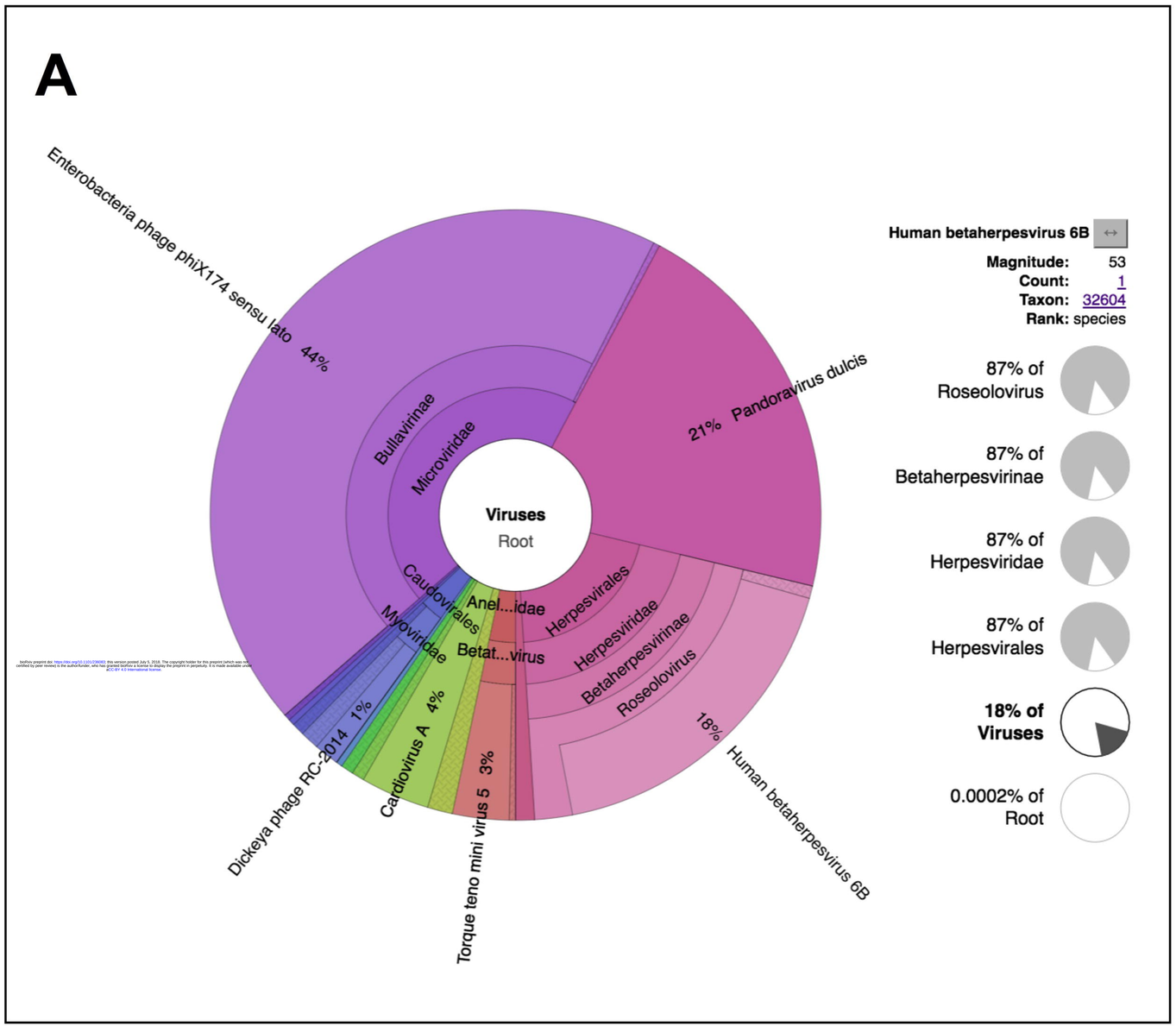
10 respiratory samples

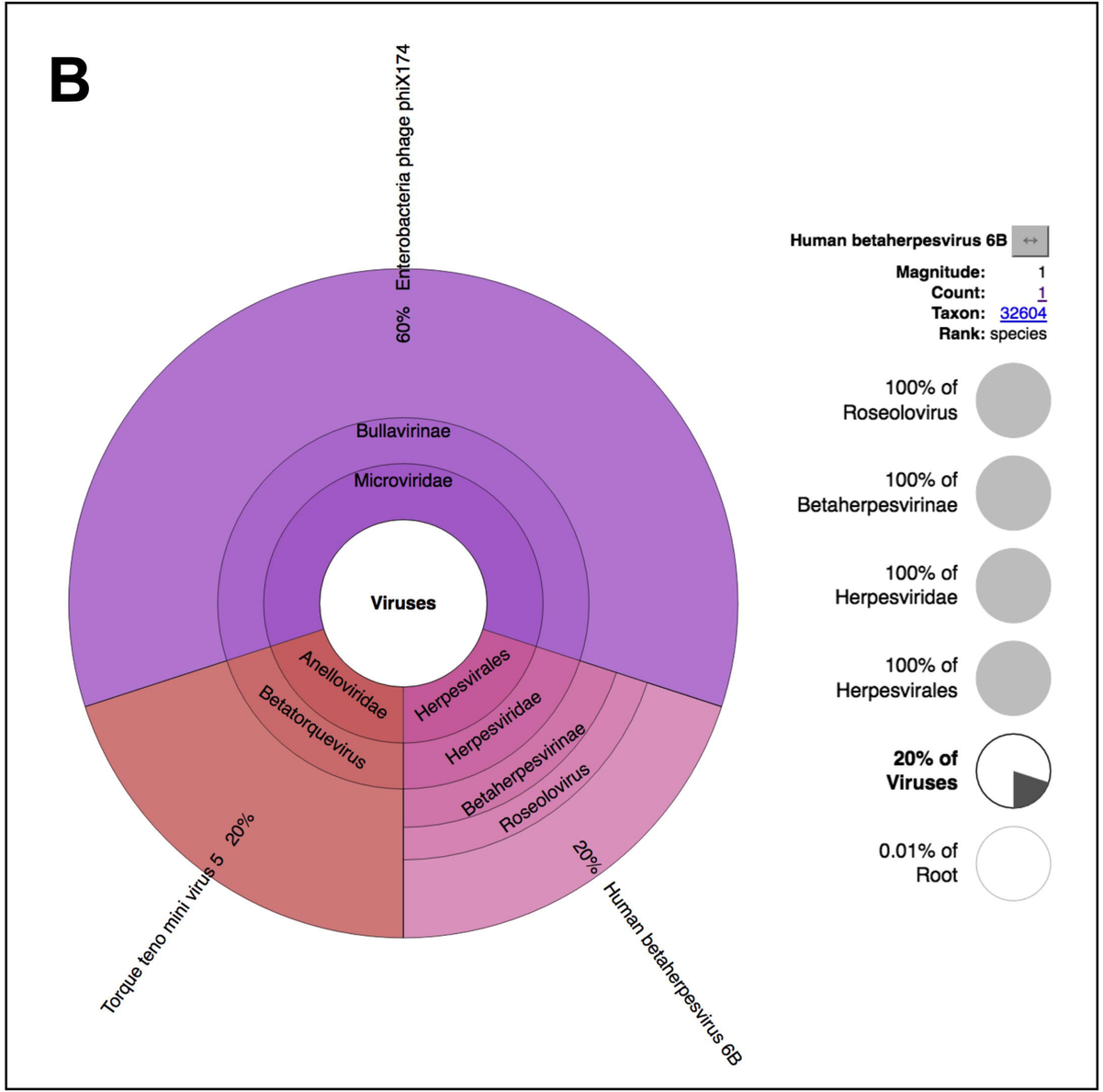
20 samples screened for HHV6 using metagenomic pipeline

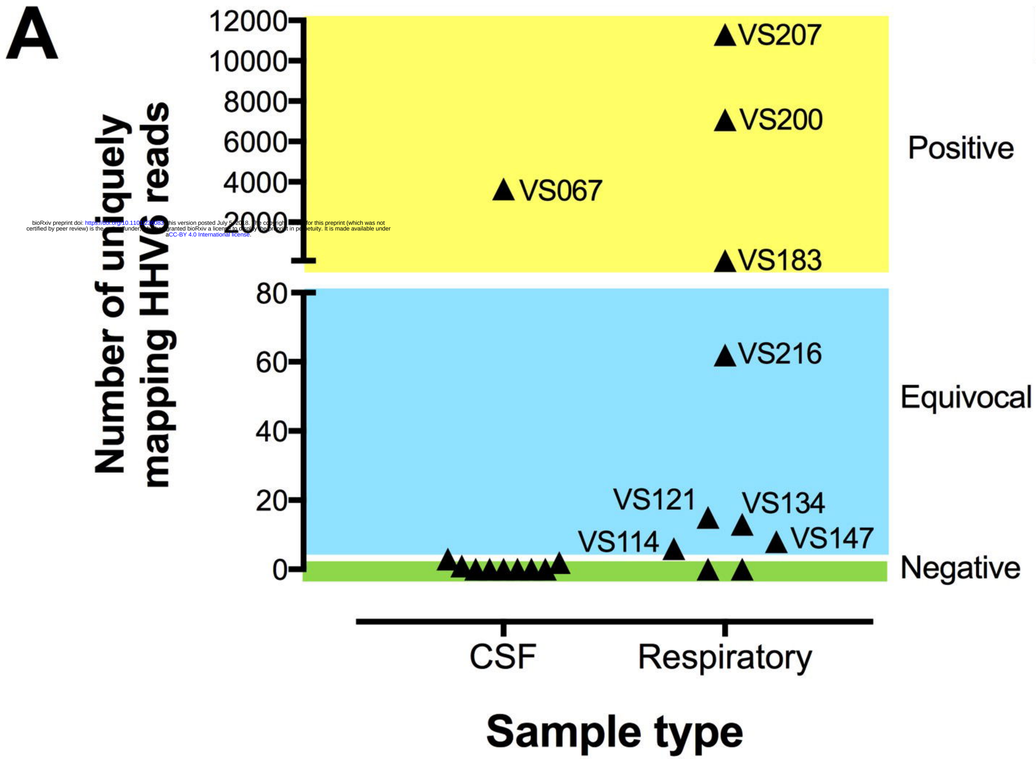
CSF samples:
1/10 (10%) HHV6 positive

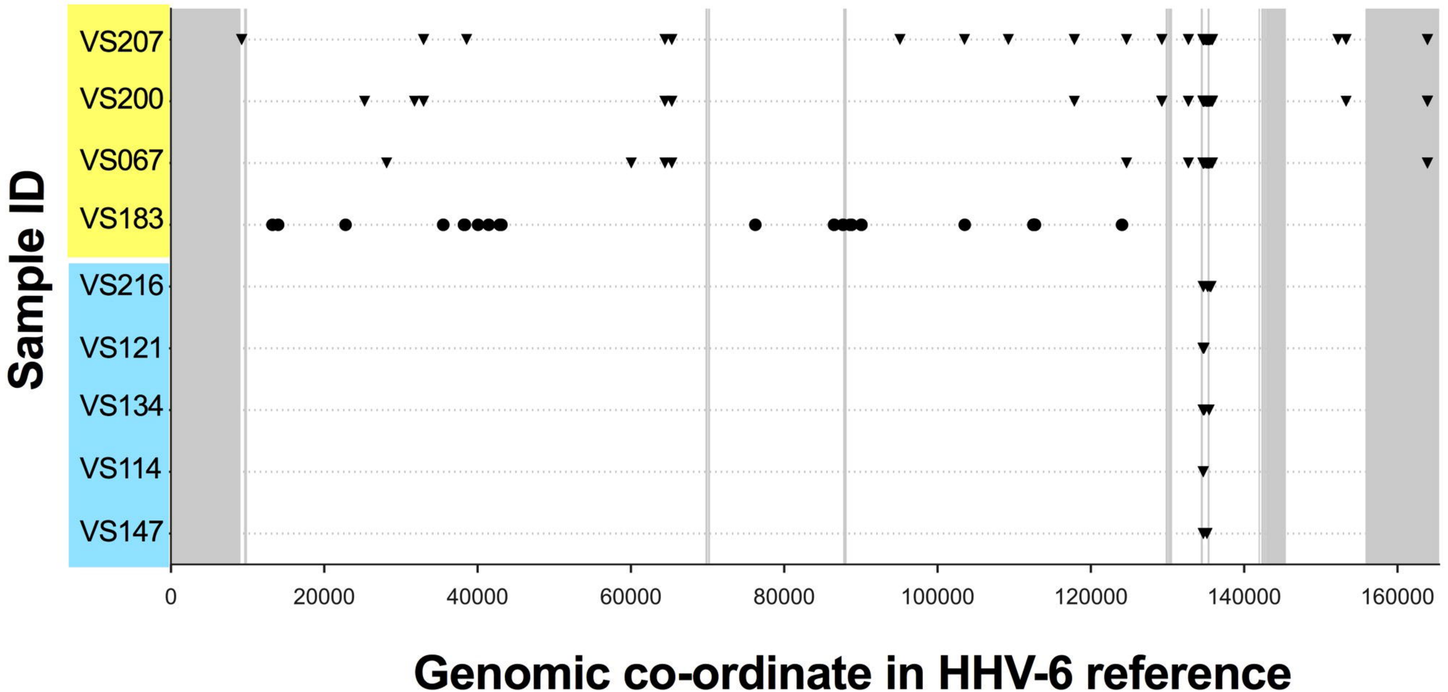
Respiratory samples:
3/10 (30%) HHV6 positive


A
p=0.05
n=36
n=5
Number of patients
40
30
20
10
0
Absent
Present
Graft vs Host Disease
HHV6-pos
HHV6-neg
B
p=0.05
n=32
n=9
Number of patients
40
30
20
10
0
Absent
Present
CNS Disease
HHV6-pos
HHV6-neg
C
p=0.03
n=13
median=870
n=5
median=290,000
HHV6 DNA viral load copies/ml
(log scale)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
Blood
CSF
Sample type
Died
Survived


A
Enterobacteria phage phiX174 sensu lato 44%
Bullavirinae
Microviridae
Viruses
Root
Caudovirales
Myoviridae
Anel...idae
Betat...virus
Herpesvirales
Herpesviridae
Betaherpesvirinae
Roseolovirus
21% Pandoravirus dulcis
18% Human betaherpesvirus 6B
Dickeya phage RC-2014 1%
Cardiovirus A 4%
Torque teno mini virus 5 3%
Human betaherpesvirus 6B
Magnitude: 53
Count: 1
Taxon: 32604
Rank: species
87% of Roseolovirus
87% of Betaherpesvirinae
87% of Herpesviridae
87% of Herpesvirales
18% of Viruses
0.0002% of Root


B
60% Enterobacteria phage phiX174
Bullavirinae
Microviridae
Viruses
Anelloviridae
Betatorquevirus
Herpesvirales
Herpesviridae
Betaherpesvirinae
Roseolovirus
Torque teno mini virus 5 20%
20% Human betaherpesvirus 6B
Human betaherpesvirus 6B
Magnitude: 1
Count: 1
Taxon: 32604
Rank: species
100% of Roseolovirus
100% of Betaherpesvirinae
100% of Herpesviridae
100% of Herpesvirales
20% of Viruses
0.01% of Root


A
Number of uniquely mapping HHV6 reads
12000
10000
8000
6000
4000
2000
80
60
40
20
0
VS207
VS200
VS067
VS183
VS216
VS121
VS134
VS114
VS147
Positive
Equivocal
Negative
CSF
Respiratory
Sample type


B
Sample ID
VS207
VS200
VS067
VS183
VS216
VS121
VS134
VS114
VS147
0
20000
40000
60000
80000
100000
120000
140000
160000
Genomic co-ordinate in HHV-6 reference

Organism(s) identified by metagenomic sequencing

Bioinformatics approach

Clinical / diagnostic microbiology interpretation

Phage / plant / animal virus?

YES

NO

Organism with definite or potential pathogenic consequences in humans?

NO

YES

Read count above a defined threshold

NO

YES

Appropriate genome coverage?

NO

YES

Confirmed ID? (e.g. by BLAST)

NO

YES

Appropriate clinical context?

NO

YES

Appropriate diagnostic sample?

NO

YES

Probable clinical, laboratory or environmental contaminant?

YES

NO

Alternative robust and plausible diagnostic result?

YES

NO

ACCEPT LIKELY DIAGNOSIS

CONSIDER

ALTERNATIVE

DIAGNOSIS