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

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31 metagenomics, next generation sequencing

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

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35

36 **ABSTRACT**

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

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

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

48 **Results:** Of 72 samples tested for HHV-6 by PCR at the request of a clinician,
49 24 (33%) were positive for HHV-6. The majority of these patients were under
50 the care of the haematology team (30/41, 73%), and there was a borderline
51 association between HHV-6 detection and both Graft versus Host Disease
52 (GvHD) and Central nervous system (CNS) disease ($p=0.05$ in each case).
53 We confirmed detection of HHV-6 DNA using NGS in 4/20 (20%) CSF and
54 respiratory samples.

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

61

62 **ABBREVIATIONS**

- 63 • cDNA – complementary DNA
- 64 • CiHHV-6 – Chromosomally integrated HHV-6
- 65 • CMV – Cytomegalovirus
- 66 • CNS – central nervous system
- 67 • CSF – cerebrospinal fluid
- 68 • EPR – Electronic patient record
- 69 • GvHD – graft versus host disease
- 70 • HHV-6 - Human herpes-virus-6
- 71 • HLA – human leucocyte antigen
- 72 • HSCT - Haematopoietic stem cell transplant
- 73 • HSV – Herpes Simplex Virus
- 74 • HTLV – Human T cell lymphotropic virus
- 75 • NGS – Next Generation Sequencing
- 76 • OUH – Oxford University Hospitals NHS Foundation Trust
- 77 • PCR – Polymerase Chain Reaction
- 78 • RSV – Respiratory Syncytial Virus
- 79 • VZV – Varicella Zoster Virus

80

81

82 BACKGROUND

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

95

96 HHV-6 variants A and B share approximately 90% homology (16). HHV-6A
97 accounts for more CiHHV-6 (17), while HHV-6B is associated with acute
98 infection, childhood rash/fever (4), and reactivation following HSCT (16).
99 Although HHV-6A is less common in CNS disease (18), it may be more
100 aggressive when present (16). HHV-6 transmission is predominantly via
101 respiratory secretions or saliva, but can also be vertical as a result of
102 chromosomally-integrated HHV-6, 'CiHHV-6' (17, 19), leading either to
103 episodic reactivation (17), or to persistent viraemia (typically $\geq 5.5 \log_{10}$
104 copies/ml (~300,000 copies/ml) in blood (20)).

105

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

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

- 132 i. Samples submitted to the laboratory by clinicians with a request for
133 **HHV-6** screening (ID numbers prefixed **HHV**). This is undertaken at the
134 request of the clinical team when deemed clinically relevant;
- 135 ii. Samples which had no request for HHV-6 testing, but had completed
136 routine diagnostic laboratory testing for other indications and were
137 used for viral sequencing studies (ID numbers prefixed **VS**).

138

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

144

145 ***Collection of local laboratory data (HHV cohort)***

146 We undertook an electronic search of the OUH Microbiology laboratory
147 system to identify all instances of an HHV-6 test (antibody or viral load) being
148 requested over three years commencing 1-Jan-2013, and recorded age, sex,
149 sample type and patient location. We used the Electronic Patient Record
150 (EPR) to determine underlying diagnosis. Follow-up data for survivors were
151 available for a median of 25 months (range 316 – 1374 days). Diagnostic
152 tests were undertaken by in-house real time PCR assays (27) at two different
153 National Reference Laboratories (Colindale up to 1-Oct-2014, and
154 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.

180

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

191

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

194

195 **RESULTS**

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

197 During the three-year study period, our clinical laboratory received 85
198 samples for HHV-6 testing (Fig 1A; suppl data set 1). In total, 41 patients were
199 tested; 22 M:19 F; median age 52 years (range 2-71; IQR 39-61). Central
200 nervous system (CNS) disease (encephalitis, encephalopathy, meningism,
201 seizures) was present in 9/41 (22%). Most patients were under haematology
202 care (30/41; 73%), of these, 5/30 (17%) had Graft versus Host Disease
203 (GvHD). 72/85 samples were tested for HHV-6 DNA by PCR at the reference
204 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 $>1.0 \times 10^6$ copies/ml in blood and 2.0×10^8 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 (9×10^4 DNA copies/ml vs. 3×10^5 copies/ml, respectively). This patient had limbic

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

232

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

238

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

240 We screened CSF ($n=100$) and respiratory ($n=100$) samples for herpesvirus
241 DNA by PCR and sequencing (Fig 1B; Suppl data set 1), identifying HHV-6 in
242 3/100 CSF samples and 5/100 respiratory samples (Table 2). Four of the
243 eight positive cases were age <5 years, representing the age group in whom
244 primary HHV-6 infection is most likely. However, in four samples (two adults
245 and two children) an alternative pathogen was identified by the clinical lab
246 (Table 2), illustrating the difficulty in distinguishing between HHV-6 as a
247 primary pathogen, a co-infecting agent contributing to pathology, or an
248 innocent bystander.

249

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

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

257

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

259 We screened a subset of 20 samples (10 CSF and 10 respiratory; Fig 1B)
260 using a metagenomic (NGS) approach (30), identifying HHV-6 in four samples
261 that we deemed 'positive'; one CSF and three respiratory samples (Table 3;
262 Fig 3). The number and distribution of reads in each sample is shown in Fig
263 4A. Sample VS183 was designated HHV-6B by Kraken, while the other three
264 positive samples were identified as HHV-6A (Fig 4B). The coverage of the
265 HHV-6 genome was incomplete, but multiple reads distributed across the
266 genome (Fig 4B), add confidence to the conclusion that HHV-6 DNA is
267 genuinely present in these samples. The sequence data have been uploaded
268 to European Nucleotide Archive (ENA) <https://www.ebi.ac.uk/ena>; HHV6
269 sequence accession numbers ERS1980462 (sample ID VS067),
270 ERS1980463 (VS183), ERS1980464 (VS200) and ERS1980465 (VS207).
271 Links to the full metagenomic sequence set can be found in our supporting
272 Data Note (30).

273

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

279

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

286

287 **DISCUSSION**

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

304

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

316
317 For samples with low HHV-6 copy numbers, PCR is anticipated to be more
318 sensitive than NGS; this is illustrated by two samples that tested positive by
319 PCR but were equivocal by NGS. However, the overall proportion of samples
320 testing positive for HHV-6 DNA was higher by NGS than by PCR. This may at
321 least in part be accounted for by PCR using highly degenerate herpesvirus
322 primers, followed by sequencing primers that have a higher degree of
323 sequence homology for HHV-6B than HHV-6A (28).

324
325 In screening by NGS, we identified HHV-6 in 4/20 (20%) samples, using a
326 combination of read numbers and genome coverage to infer positivity (33).
327 Multiple considerations feed into the interpretation of NGS data (Fig 5). The
328 four patients positive for HHV-6 by NGS in this cohort all had clinical
329 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

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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

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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

378 **AUTHORSHIP**

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

384

385 **FIGURE LEGENDS**

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

396

397 **Figure 2: Results of clinical laboratory testing for HHV-6 in blood and**
398 **CSF samples at the request of the clinical team.** A: Relationship between
399 presence or absence of Graft vs Host Disease (GvHD) and HHV-6 status; P
400 value by Fisher's exact test. B: Relationship between presence or absence of
401 a clinical Central Nervous System (CNS) syndrome and HHV-6 status; P
402 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

449

450 **Table 1: Relationship between HHV-6 status and clinical/demographic**
 451 **features in 41 patients with samples submitted for HHV-6 testing to a**
 452 **clinical UK microbiology lab.**
 453

	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

454

455 ^a In the case of patients tested more than once, any instance of PCR positive
 456 is recorded as positive; ^b Sample types were not documented for 3 patients
 457 testing PCR-positive, and for 7 patients testing PCR-negative; ^c CNS=central
 458 nervous system; ^d Prospective survival data were available for 40/41 patients.
 459

460

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/ μ l)	CSF cell count: lymphs (cells/ μ l)	CSF cell count: RBC (cells/ μ l)	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	? Bronchiolitis	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	<i>Streptococcus viridans</i>	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 Lymphocyte count (cells/ μ l)	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)

478

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

484

485

486

487 SUPPLEMENTARY RESOURCES

488

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

492 This fileset includes the following:

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

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667

A **B**

A **Graft vs Host Disease**

HHV6-pos
HHV6-neg

B **CNS Disease**

HHV6-pos
HHV6-neg

C **Sample type**

Died
Survived

A

B

A **B**

Organism(s) identified by metagenomic sequencing

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

Bioinformatics approach

Clinical / diagnostic microbiology interpretation