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

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ABSTRACT

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. In order to determine patterns of HHV-6 infection and detection, we took two approaches. First, we scrutinized HHV-6 requests in samples submitted to our UK hospital laboratory through routine diagnostic pathways. 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 (73%), and there were borderline associations between testing positive for HHV-6 DNA and both Graft versus Host Disease (GvHD) and central nervous system (CNS) disease (p=0.05 in each case). Second, we undertook a pilot study using Illumina next generation sequencing to investigate the application of this tool to detection of HHV-6 in CSF and respiratory samples that were initially submitted to the laboratory for other diagnostic tests. We confirmed detection of HHV-6, but highlight challenges in interpretation of metagenomic data and the need for further controls to define sensitivity and specificity. Our results lead to recommendations for the improvement of existing laboratory protocols, and underpin ongoing efforts to explore next generation sequencing 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

INTRODUCTION

Human herpes-virus-6 (HHV-6) is a human beta-herpesvirus, first described in 1986 in patients with Human T cell lymphotrophic virus (HTLV) infection and/or haematological malignancy (1). Like its close relative human cytomegalovirus (CMV), it is ubiquitous (frequently infecting >95% of the population by the age of two years), 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, clinical consequences of HHV-6 range from a trivial (or asymptomatic) infection, to a more significant febrile illness with rash ('roseola'), or occasionally encephalitis (6). HHV-6 reactivation has also been described in the setting of encephalitis or meningitis caused by other pathogens, such as enteroviruses, in which context it appears to follow a generally benign course (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 bone marrow transplantation (9-15).

HHV-6 transmission is predominantly via respiratory secretions or saliva, but can also be vertical. The majority of mother-to-child transmission occurs as a result of high-level germline integration, referred to as chromosomally-integrated HHV-6, 'CiHHV-6' (16, 17). This chromosomal integration, present in an estimated 1% of the population, can lead either to reactivation (17), or to persistent HHV-6 viraemia, with a suggested threshold of ≥5.5 log10

copies/ml (approx. 300,000 copies/ml) being indicative of CiHHV-6 (18). HHV-6 has two variants, A and B, that share approximately 90% sequence homology (19). HHV-6A accounts for more CiHHV-6 (17), while HHV-6B is more likely to be acquired as an acute infection, is associated with childhood rash/fever (4) and with reactivation following bone marrow transplantation (19). Although HHV-6A is less common as a cause of CNS disease (20), it may be more aggressive when present (19).

Given that exposure to the virus is common, HHV-6 laboratory diagnostics raises a number of challenges: what sample type to test, in which patient groups to focus, and how to should interpret a positive test result. There is increasing interest in metagenomic ('next generation sequencing', NGS) approaches to clinical microbiology diagnosis of many pathogens (21-25) but ongoing development is required to optimize these pipelines, including tackling the challenge of high proportion of human DNA reads, differentiating between pathogenic organisms and commensal or environmental flora, and determining thresholds at which the identification of an organism is likely to be clinically significant (26) (Suppl data set 1). The latter challenge is particularly pertinent for herpes viruses, which are ubiquitous and potentially integrated into the host.

We here present data first from the local diagnostic microbiology laboratory, to review the patterns of clinical testing for HHV-6, and to describe the patients who test HHV-6 DNA positive. Second, we report the results of screening CSF and respiratory samples, in cases where HHV-6 testing had not been

specifically requested, using PCR and NGS in a small pilot study to ascertain the extent to which this virus can be detected in routine diagnostic 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 detection of this organism.

MATERIALS AND METHODS

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:

i. Samples submitted to the laboratory by clinicians with a request for **HHV-6** screening (study ID numbers prefixed by **HHV**); the metadata file for this cohort is provided on-line (Suppl data set 1).

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 (study ID numbers prefixed by **VS**); supporting datafiles for metagenomic work are provided online (Suppl data set 1).

Approval for retrospective collection of clinical and laboratory data was granted by the Oxford University Hospitals NHS Foundation Trust Clinical

Audit Committee (HHV cohort samples). Testing of consecutive anonymised laboratory samples (VS cohort samples) 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 a three year period commencing 1-Jan-2013, and recorded age, sex, sample type and patient location. We interrogated the hospital Electronic Patient Record (EPR) to determine the patient's underlying diagnosis. Follow-up data for survivors were available for a median period of 25 months (range 316 – 1374 days). During the period of the study, diagnostic tests were sent to two different National Reference Laboratories (Colindale from 1-Jan-2013 to 1-Oct-2014, and Bristol for the remainder of the study period). Testing in these centres was undertaken by in-house real time PCR assays (27).

Data collection and statistical analysis

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

Testing CSF and respiratory samples (VS cohort)

We identified 100 CSF samples and 100 respiratory samples (comprising throat swabs (n=22), nasopharyngeal aspirates (n=42), endotracheal aspirates (n=4), and bronchoalveolar lavage samples (n=32)) that had been processed by the diagnostic microbiology laboratory in 2013/2014. These represented a subgroup of patients deemed to be at 'high risk' of viral infection based on the following criteria:

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

Samples had all undergone clinical laboratory testing as described in our supplementary methods file (Suppl data set 2) and were stored at -80°C prior to further processing. We selected consecutive samples; the only exclusion criterion was inadequate sample volume (<200 ul). For each sample, we documented patient age group and clinical location, and recorded the clinical information that had been supplied with the sample and microbiology laboratory data generated as part of the primary clinical testing pipeline.

PCR for HHV6 (VS cohort)

Additional experimental testing was undertaken at the Roslin Institute, University of Edinburgh. 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 directly 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).

Next generation sequencing using Illumina HiSeq and Bioinformatic analysis of NGS dataset (VS cohort)

Our methods are described in supplementary data files, available on-line (Suppl data set 2).

RESULTS

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

During a three-year study period, our clinical laboratory received 85 samples with a request for HHV-6 testing (Fig 1A). In total, 41 different patients were tested; 22 M:19 F; median age 52 years (range 2-71; IQR 39-61). For full clinical dataset see Suppl data set 1. Most patients were under the care of the haematology team (n=30; 73%), among whom five had Graft versus Host Disease (GvHD). Nine patients had a report of central nervous system (CNS) disease, including encephalitis, encephalopathy, meningism and seizures.

Results of HHV-6 testing (HHV cohort)

Of 85 samples received, 72 were tested by PCR at the regional reference laboratory, 24 were positive for HHV-6 DNA (33%). Among 41 patients tested, 15 (37%) were PCR positive at some point during the three-year time period under review (Fig 1A). Although subtyping is not now undertaken routinely, of seven samples that were subtyped, all were reported as HHV-6B. Among patients who tested PCR positive, this was more frequently derived from blood (9/15 patients) than from CSF (3/15 patients) (Table 1). Positive HHV-6 status was not statistically associated with patient age, sex, or haematological malignancy, but there was a borderline association with both GvHD and CNS disease (p=0.05 in both cases; Table 1; Fig. 2A and B). On multiple logistic regression analysis, there was no relationship between HHV-6-positivity and any patient or laboratory characteristic (Table 1).

In this cohort, 23/41 (56%) of patients died, at a median age of 56 years. Of those who died, 11/23 (48%) had tested HHV-6-PCR positive at some point during our study period, compared to surviving patients of whom 4/18 (22%) had a positive HHV-6 PCR, although this difference did not reach statistical significance (p=0.1). All those who died did so within 19 months of the HHV-6 test (range 1-552 days, median 163 days). We did not have sufficient clinical data to determine cause of death.

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

HHV-6 DNA quantification was undertaken in 23 samples from ten different patients (Fig 2C). The levels varied from below the threshold for accurate quantification in serum and bone marrow, to an individual with $>1.0 \times 10^6$

copies/ml in blood and 2.0×10^8 DNA copies/ml in CSF (patient ID HHV-012), suggesting CiHHV-6. Another patient had HHV-6 DNA detected in both a blood and CSF sample (patient ID HHV-007, 9×10^4 DNA copies/ml in blood vs. 3×10^5 copies/ml in CSF). This patient, an adult with haematological malignancy, was reported to have 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.

Longitudinal testing (HHV cohort)

Among a total of 72 tests, 46 were longitudinal samples from thirteen individual patients. Among these thirteen, the median number of serial tests was three, but one individual (ID HHV-045) had seven EDTA blood samples and a CSF sample tested within an 8 week time period (of these, only one blood sample was positive for HHV-6 DNA). There was no statistical association between multiple HHV-6 tests and other factors that might predict disease severity (age, sex, mortality, haematological malignancy, transplant, intensive care location, documented co-infection; $p>0.1$ for all parameters in multiple regression analysis).

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

We screened consecutive laboratory CSF (n=100) and respiratory (n=100) samples for herpesvirus DNA by PCR, and subsequently confirmed HHV-6 by sequencing (workflow shown in Fig 1B, and Suppl data set 1). We identified HHV-6 DNA in eight (4%) of these, comprising three CSF samples and five respiratory samples (the samples testing PCR positive are shown in Table 2).

Four of these PCR-positive cases were children age <5 years, representing the age group in whom primary HHV-6 infection is most likely to occur. However, in four samples (two from adults and two from children) an alternative potential pathogen was identified by the clinical diagnostic lab (VZV, HSV, Rhinovirus, Metapneumovirus). This illustrates the difficulty in distinguishing between HHV-6 as a primary pathogen, as a co-infecting agent contributing to pathology, or as an innocent bystander (potentially as a result of reactivation).

The overall HHV-6 prevalence of 4% in this group of unselected samples is significantly lower than the 33% rate obtained from the samples in which the clinician had specifically 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 an unwell hospitalized population.

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. In these 20, we identified HHV-6 in four samples that we deemed 'positive'; one CSF and three respiratory samples (Table 3). The representation of HHV-6 reads as a proportion of the virome is shown for one representative respiratory sample in Fig 3, and the

number and distribution of reads in Fig 4. Sample VS183 was designated HHV-6-B by Kraken, while the other three positive samples were identified as HHV-6-A (Fig 4B). The coverage of the HHV-6 genome was only 1% in each case, but multiple reads distributed across the genome, including in conserved regions (Fig 4B), add confidence to the conclusion that HHV-6 DNA is genuinely present in these samples. The equivocal outcomes, comprising low numbers of HHV-6 reads, were all respiratory samples, and the sequences clustered in the repeat region of the genome, suggesting lower specificity for HHV-6.

In sample VS067 the clinical syndrome was not explained by other diagnostic results, and HHV-6 infection was a plausible agent of the clinical syndrome (in keeping with 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.

The metagenomic 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 (sample ID VS183), ERS1980464 (sample ID VS200) and ERS1980465 (sample ID VS207).

Concordance between PCR and NGS (VS cohort)

Among the total of eight samples that were HHV-6 positive by conventional PCR, three were tested on the NGS pipeline (sample IDs 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

Summary of findings

This preliminary study provides insight into the patterns of HHV-6 testing in a large UK teaching hospital, with the aim of updating recommendations for consistent laboratory practice and translating this into improvements in clinical practice. We also describe the application of two different approaches (targeted PCR, vs. a sequence-independent NGS pipeline) to report the 'background' presence of HHV-6 DNA in CSF and respiratory samples at the point of discard from a routine clinical microbiology laboratory. Although our NGS data only represent a small pilot study, to date, we have identified only a single other reference that describes a metagenomic approach to the diagnosis of HHV-6 infection (6).

Although it is recognized that HHV-6 can reactivate in the context of critical illness (e.g. septic shock (8)), we here found a higher prevalence in clinical samples taken from a high-risk group (HHV cohort) that in laboratory samples representing an unwell and immunocompromised hospital population (VS cohort), suggesting that HHV-6 is not simply reactivating across the board in unwell hospitalized patients. Although we cannot attribute mortality to HHV-6

in the context of this study, a recent study documents poor outcomes of HHV-6 disease in the bone marrow transplant population (15).

Implications for HHV-6 screening and treatment

The combination of a high rate of HHV-6 DNA detection and the high mortality (HHV cohort), suggest that we should consider lowering our threshold for testing in this context, in order to identify patients who may benefit from treatment earlier in the course of disease. In future, enhanced prospective surveillance may help shed more light on the clinical significance of HHV-6 in patients with haematological malignancy. This study was not designed to evaluate the use or outcome of antiviral therapy as this information could not robustly be captured retrospectively for the time period in question. There are some data to suggest good outcomes from treatment of symptomatic viraemia (30), most commonly 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 (31); 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.

Suggested applications for current clinical diagnostic pipeline

Our recommendations following this study include:

i. Diagnostic laboratories should only offer testing for HHV-6 PCR, not for IgG testing. The rationale for this is that the majority of individuals are likely to have been exposed, and therefore will be HHV-6 IgG positive.

In the setting of profound immunocompromise, where there may be a wish to identify patients at risk of reactivation, IgG may be falsely negative due to the reduced pool of productive B cells.

ii. Longitudinal HHV-6 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 for diagnostic purposes may be helpful.

Significance of HHV-6 subtype

Currently, HHV-6 subtype has no consistent bearing on the approach to clinical management and is therefore not part of routine testing. However, further studies, including NGS, may determine whether different clinical syndromes are consistently associated with the two subtypes. In this study, all viruses subtyped by the clinical laboratory were HHV-6B, which reflects the anticipated pattern. In contrast, the NGS primarily detected HHV-6A, potentially reflecting reactivation of integrated virus.

Sensitivity and specificity of HHV-6 detection by different methods

Assigning a sensitivity or specificity to different methods is difficult in the absence of a single gold standard test that includes application to metagenomic data. For samples with low HHV-6 copy numbers, PCR is anticipated to be more sensitive than the current metagenomic approach; 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 use of highly degenerate herpesvirus primers that were used to amplify DNA, followed by sequencing primers that have a higher degree of sequence homology for HHV-6B than HHV-6A (28). Although we cannot exclude the possibility of cross-contamination by samples on the HiSeq platform, this is more frequently encountered in the context of samples with high viral loads, and the low copy numbers in our samples generally make this unlikely.

Clinical significance of NGS data

In screening unselected samples by NGS, we identified HHV-6 at levels deemed to be potentially clinically significant in 4/20 (20%) samples, although further studies are needed, and these thresholds remain to be determined with confidence. Importantly, positivity cannot be inferred by read numbers alone (32), and we have also used genome coverage to help infer which samples were truly positive for HHV-6. The four patients positive for HHV-6 by NGS 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. When HHV-6 detection is deemed significant, this could support the introduction of antiviral therapy, and/or reduce exposure to broad-spectrum antibiotics.

In parallel with wet lab methods, bioinformatic approaches for interpreting metagenomic data derived from clinical samples are still evolving. Multiple considerations feed into this interpretation, some of which we have summarized as an algorithm in Fig 5. More robust approaches will evolve as the volume and quality of data from clinical metagenomic testing improves.

Caveats and limitations of this study

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. Together, these factors reduce statistical power in the identification of significant risk factors or clinical associations of HHV-6. Our data quality is dependent upon the completeness of electronic data, and we may have under-represented certain clinical groups due to lack of complete information. For our VS cohort, we screened only CSF and respiratory samples; inclusion of blood samples remains an important avenue for future enquiry.

Further evaluation of optimum methods for NGS is required; some of the potential caveats are presented in more detail in our supplementary methods file (Suppl data set 2). For our own future metagenomic work, we are currently developing positive internal controls, and for HHV-6 specifically, an international control standard is currently being prepared by the National Institute for Biological Standards and Control (NIBSC).

Summary

This study improves our understanding of HHV-6 detection both in a clinically selected population and in a cross-section of laboratory samples. On the basis of the clinical component of the study, we have improved the consistency of our laboratory approach to these requests. 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 aged <5 years. Metagenomic approaches to clinical diagnostics are accelerating, and as additional data become available, increasing insights will be gained into the interpretation of these results.

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.

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

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.

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 by Mann Whitney test; individuals who died are shown in red. In all three panels, the numbers at the top of each column show the total number of patients represented.

Figure 3: Multi-layered pie charts generated to visualize the metavirome from a respiratory sample taken from a child. Krona was used to generate the metagenomic visualizations of these data (34). The sample was a

nasopharyngeal aspirate taken from patient ID VS183 (a child age <5 with a clinical syndrome described by the requesting clinician as 'viral infection'). (A) HHV-6 B (in pink) shown as a proportion of all viral reads; (B) HHV-6-B (in pink) shown as a proportion of all virus contigs. The other two predominant viruses represented in both panels are Torque Teno Mini Virus (in red), a ubiquitous and probably non-pathogenic virus, and Enterobacteria phage Phi X (in purple), which is an artefact of the sequencing method (spike used for positive control in Illumina sequencing run); these illustrate a high proportion of sequence reads generated from organisms that are not clinically significant.

Figure 4: Detection of HHV-6 DNA in unselected CSF and respiratory samples. A: Read numbers determined by next generation sequencing (NGS) according to sample type. The y-axis refers to the number of uniquely mapping HHV6 reads, representing deduplicated read numbers (Q>30). The areas shaded green, blue and yellow represent suggested thresholds for samples to be classified as negative, equivocal or positive, respectively. White gaps between the colours illustrate that the exact position of the boundaries between these areas are uncertain. B: Plots generated with Burrows-Wheeler Aligner (BWA) to illustrate coverage of HHV-6 genomes against consensus. The x-axis of the plot represents the full length genome for HHV-6, with HHV-6A shown in triangles, and HHV-6B shown in circles. From a total of 20 samples tested, we show data for samples from which we generated any HHV-6 reads. The beginning position of each read is indicated. The grey bars indicate repeat regions (low variability) as defined in the methods; for a

sample to be considered positive, we stipulated that the reads should fall outside these regions of the genome.

Figure 5: Suggested algorithm showing process of determining the significance of an organism identified from a clinical sample by next generation sequencing (NGS). This represents a structure that can be applied to bioinformatics and clinical interpretation of metagenomic data. We recognize that the approach and thresholds are different for different organisms, and that robust output also depends also on optimization of *in vitro* sample preparation.

SUPPLEMENTARY RESOURCES

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

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.

Suppl data set 2. Oxford Screening of CSF and Respiratory Samples ('OSCAR'): Supporting data files for a project using Next Generation Sequencing (NGS) for identification of viruses from clinical laboratory samples; DOI 10.6084/m9.figshare.5670007

This fileset includes the following:

- OSCAR protocol as .pdf file (detailed laboratory methods);
- OSCAR table benefits and challenges (summary of benefits and challenges of NGS approach to clinical samples);
- OSCAR 20 samples metadata (subset of 20 samples from VS cohort for which next generation sequencing was undertaken) as .xlsx and .csv files.

Data note in submission to BMC Research Notes

This short note, currently in submission, comprises an overview of the metagenomic screening project presented here, with links to the methods (as presented above in suppl data set 2) and to the complete dataset in two formats as follows:

- An interactive html file (Krona (34))
- Metagenomic sequences uploaded to European Nucleotide archive (35)

A citation / DOI will be added to this manuscript on acceptance by BMC Research Notes.

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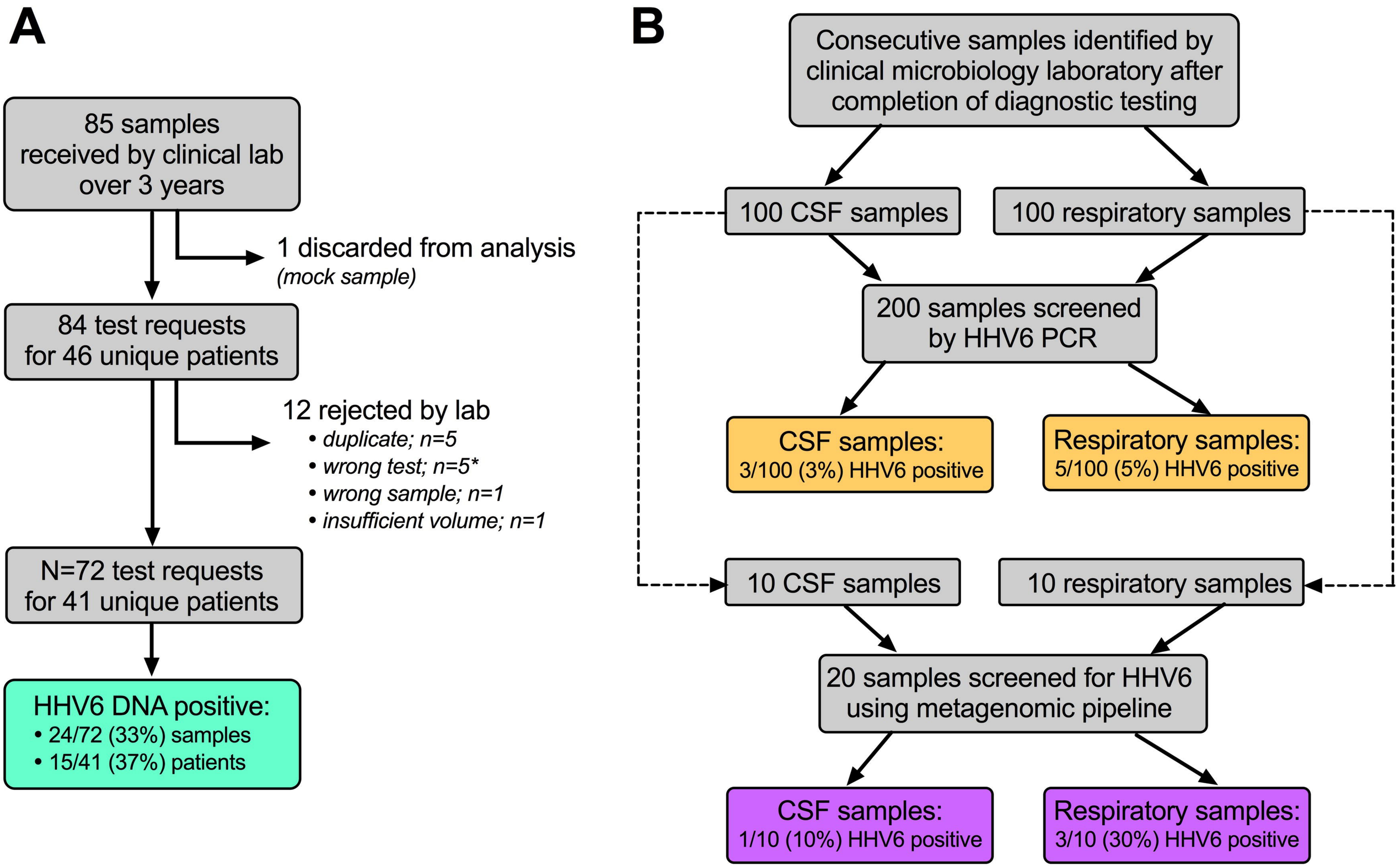
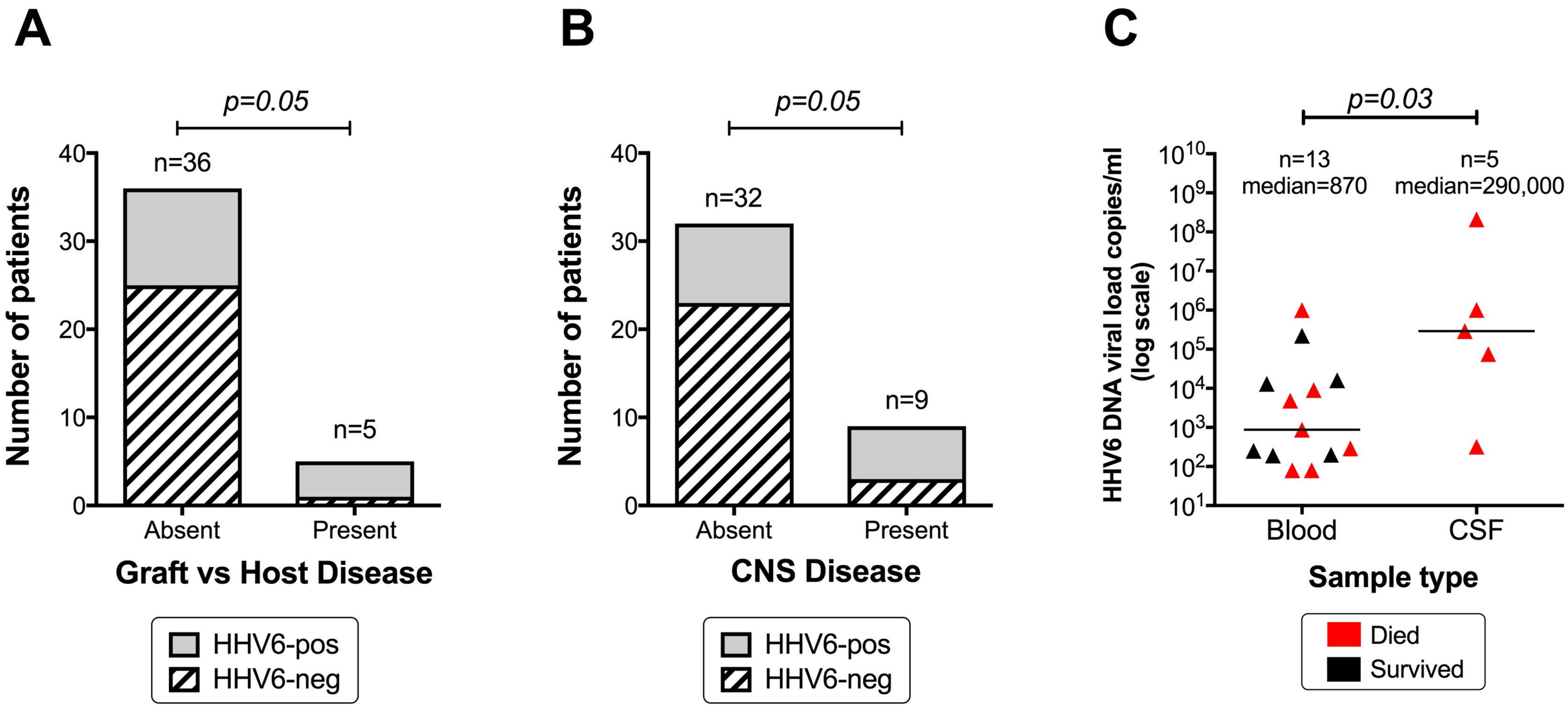
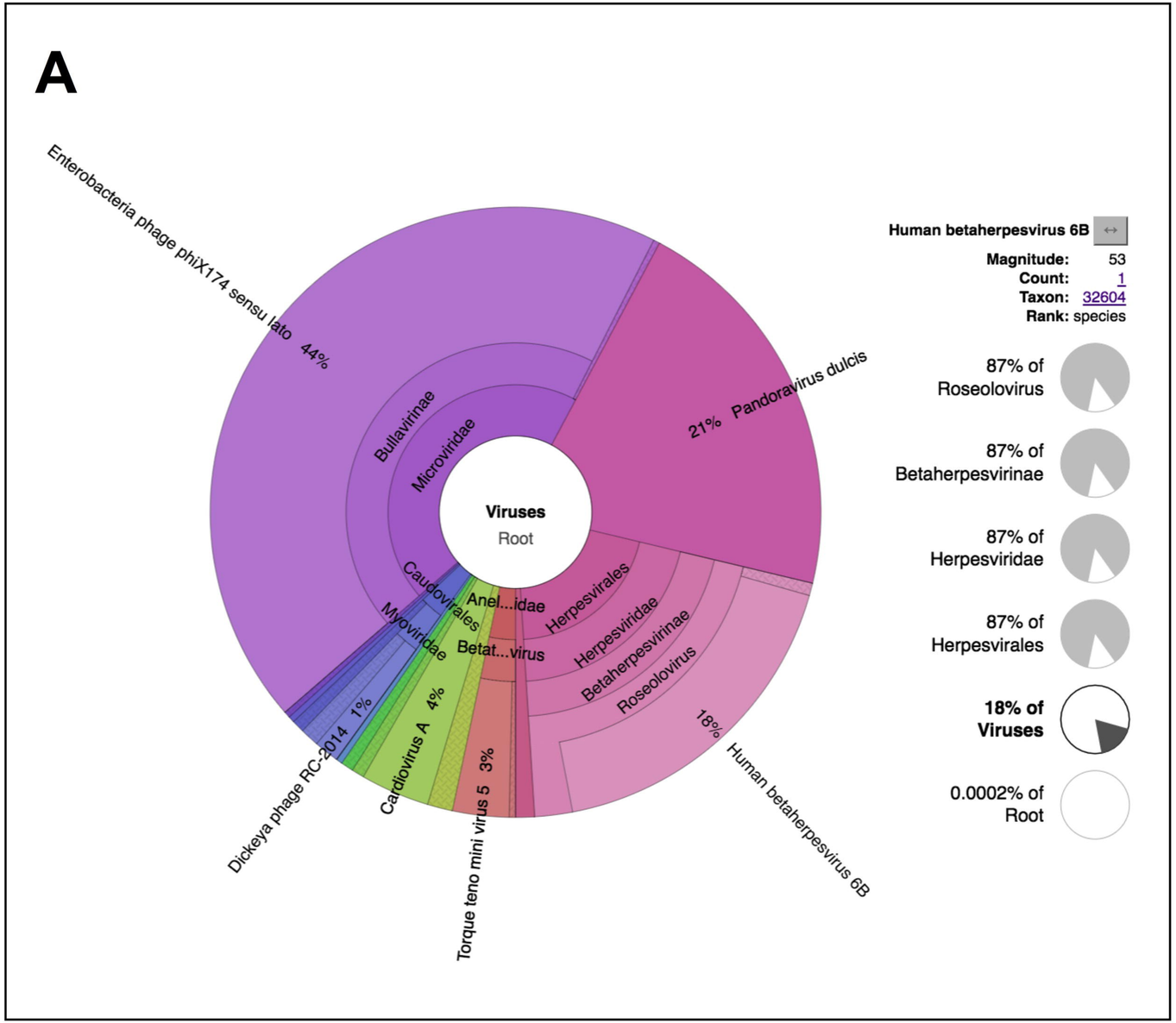
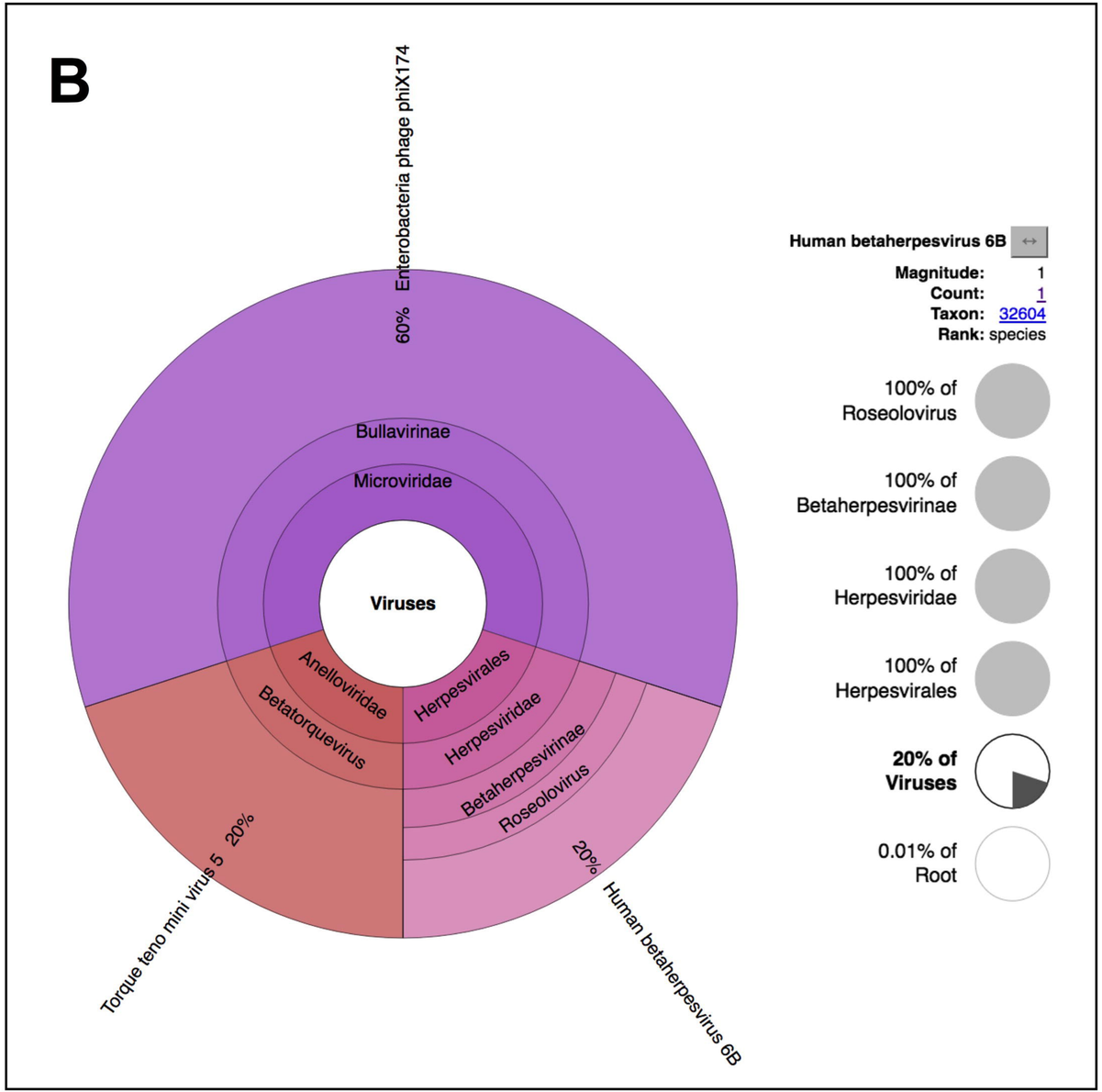
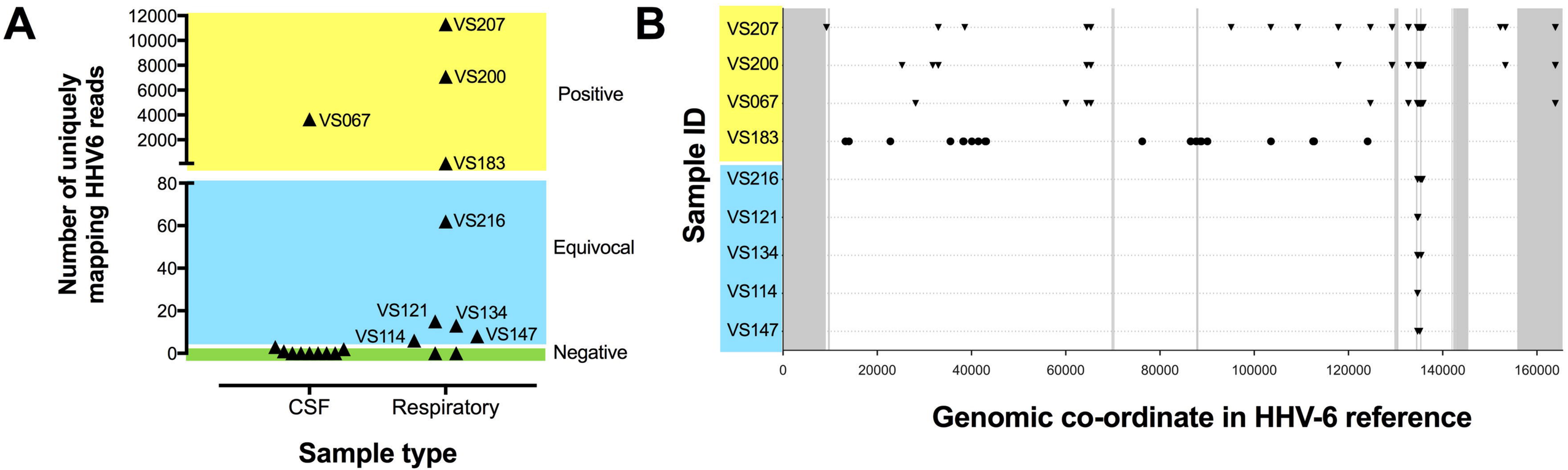
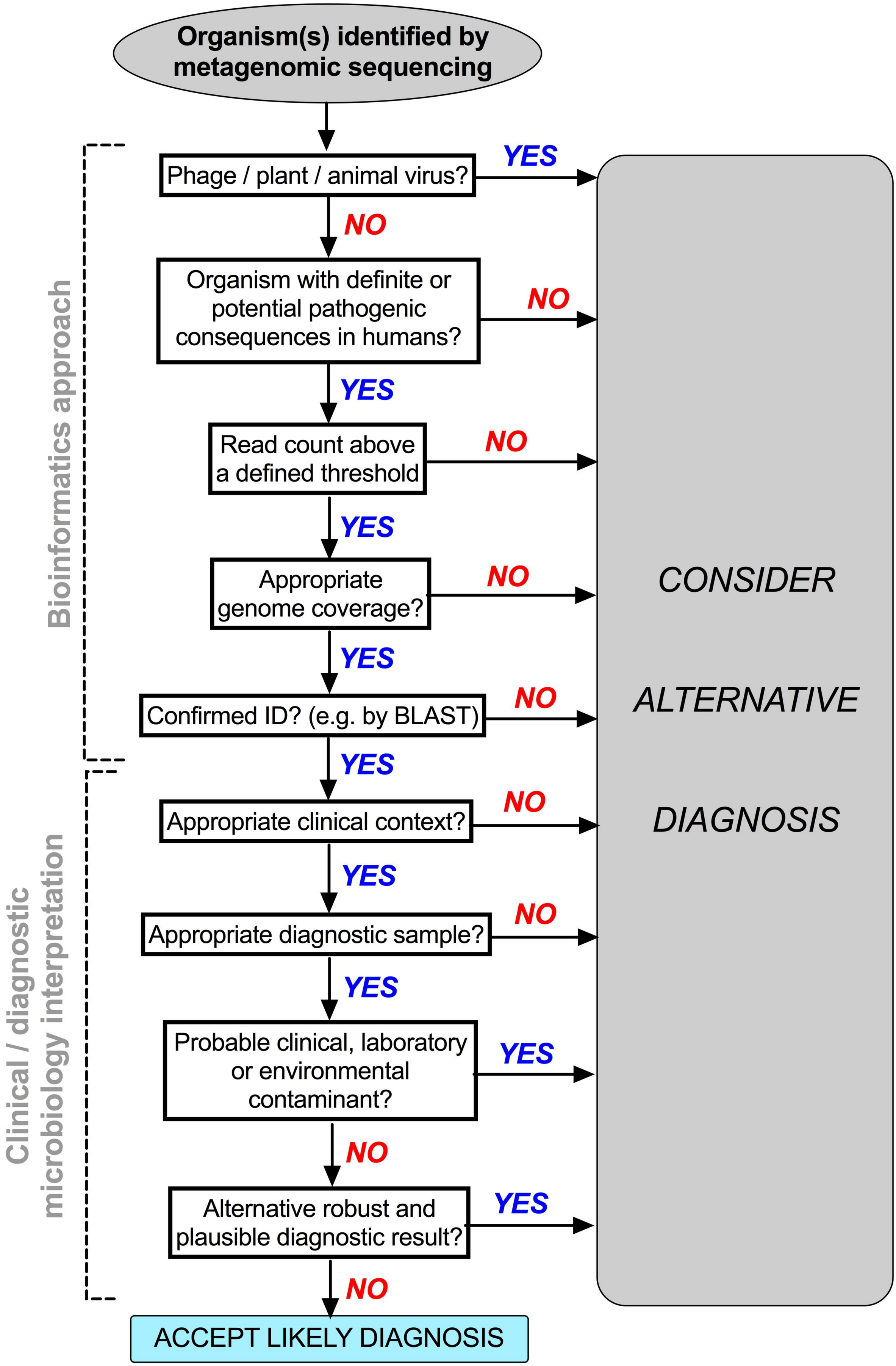

A
85 samples received by clinical lab over 3 years
1 discarded from analysis
(mock sample)
84 test requests for 46 unique patients
12 rejected by lab
• duplicate; n=5
• wrong test; n=5*
• wrong sample; n=1
• insufficient volume; n=1
N=72 test requests for 41 unique patients
HHV6 DNA positive:
• 24/72 (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%
Pandoravirus dulcis 21%
Human betaherpesvirus 6B 18%
Torque teno mini virus 5 3%
Cardiovirus A 4%
Dickeya phage RC-2014 1%
Bullavirinae
Microviridae
Viruses
Root
Caudovirales
Myoviridae
Anel...idae
Betat...virus
Herpesvirales
Herpesviridae
Betaherpesvirinae
Roseolovirus
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
Enterobacteria phage phiX174 60%
Torque teno mini virus 5 20%
Human betaherpesvirus 6B 20%
Bullavirinae
Microviridae
Viruses
Anelloviridae
Betatorquevirus
Herpesvirales
Herpesviridae
Betaherpesvirinae
Roseolovirus
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
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