Human Herpes Virus Type 6 is Associated with CNS Infections in Children in Sudan

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Research note

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Abstract

Objective

HHV-6 is increasingly recognized as febrile agent in children, however, less is known in sub-Saharan African countries. In here, we aimed to investigate the involvement of HHV-6 in pediatric CNS infections in Khartoum, Sudan. This report is part of a larger study on the microbial aetiologies of CNS infections in this population.

Results

Out of 503, 13 (2.6%) CSF specimens were positive for HHV-6 DNA which constituted 33% (13/40) of cases with proven infectious meningitis. Median Ct for all HHV-6 positive specimens was 38 with range of 31.9 to 40.8. Median virus copy was 281.3/PCR run (1×10^5 virus copies/ml CSF) with range of 30 to 44×10^3 copies/PCR run (12×10^3 and 18×10^6 virus copies/ml CSF). All positive patients presented with fever, vomiting and 86% with seizures. Male to female ratio was 1:1; 50% were toddlers, 42% infants and 8% teenagers. Most (83%) were admitted in the dry season and 17% in the rainy season. CSF leukocytosis was seen in 33%. Normal and low CSF glucose levels were seen in 86% and 14%, respectively. CSF proteins levels were low in 14% and high in 43%. In conclusion, HHV-6 is common in CNS infections in children in Sudan.

Introduction

HHV-6 is major cause of acute febrile illnesses in young children [1]; most are infected by age of three [2]. After primary infection, HHV-6 persists in salivary glands and remains latent in leukocytes [3]. Immunosuppression can lead to virus reactivation and various complications can occur, including CNS disease [3]. CNS disease can also occur in primary infections, especially in young immunocompetent children with fever and seizures [4, 5]. In this matter, several authors described the major role the virus could play as a cause of pediatric neuroinfections [6–11].

In the absence of primary infection, the key finding linking HHV-6 to CNS infections is detection of viral DNA in CSF by PCR; indicating active replication. This interpretation has been questioned in view of the phenomenon of HHV-6 chromosomal integration [12, 13]. HHV-6 is the only human herpes virus found integrated into human genome and can be transmitted vertically from parent to child [14]. Such individual is easily identifiable, since every leukocyte contains viral sequences and there are thus characteristically persistent high levels of HHV-6 DNA in both serum and whole blood [12, 13]. In contrast, integrated HHV-6 DNA is highly unexpected in normal cell-free body compartments, including CSF [11, 13, 15]. Ward [16] stated that viral load should be high to identify a condition as chromosomal integration and low virus copies would indicate an infection.

The outcome for HHV-6 infection is generally favorable, with most patients recovering fully. Nevertheless, mild to moderate neurological impairment as well as death due to complications has been reported [22,

23]. Little is known on HHV-6 infections in sub-Saharan Africa, and the infection has never been investigated in Sudan. We, therefore, intended to identify the involvement of HHV-6 (among other microbial aetiologies reported elsewhere) in CNS infections in large group of children in Khartoum, Sudan.

Methodology

Study Materials

Total of 503 CSF specimens were obtained from febrile ($\geq 37^{\circ}\text{C}$) meningitis-suspected attendee of Omdurman Hospital for Children in Khartoum, Sudan. Patients ages 0 to 15 years who were admitted in all seasons of 2010 were included. Clinical and demographic data were obtained from hospital records. Routine CSF analyses were performed at the Microbiology Laboratory of Omdurman Hospital. An aliquot of CSF was frozen in -80°C for further analysis for HHV-6 DNA at the department of Clinical Microbiology, Umeå University, Sweden. Permission to collect data and specimens was granted from hospital authorities. Ethical clearance was obtained from the Ethical Committee Board of Al-Neelain University.

HHV-6 DNA Analysis

QIAampUltraSensVirusTechnology (Qiagen®) was used to extract viral DNA in 1 mL CSF following the manufacturer recommendations. Pure viral nucleic acids were eluted in 30 μL low-salt buffer AVE twice. Each elute (60 μL) was divided into two aliquots and preserved at -80°C .

Viral gDNA amplification and detection was performed by real-time analysis using Applied Biosystems®7900HT Fast Real-Time PCR system and TaqMan® probe (reporter dye FAM™ on 5' end and quencher dye TAMRA™ on 3' end). Forward and reverse primers (Table S1) and probes (DNATechnology®) were diluted to reach final working concentration of 25 μM (standardized concentration by Umeå University Hospital). Commercially provided oligonucleotide products were diluted to the suitable working solution and the recommendation of QuantiTect®QPCR Protocol was followed. Master mix was prepared for HHV-6 primers as follow: 12.5 μL of 2x QuantiTect™ Probe PCR Master Mix (Qiagen®), 1.0 μL forward primer (final conc. 25 μM), 1.0 μL reverse primer (final conc. 25 μM), 0.8 μL probe (final conc. 20 μM) and 7.2 μL RNase-free water (Ambion®) were added into 2.5 μL template gDNA to complete total reaction volume of 25 μL per single PCR reaction. The mixture was pulse-vortexed and centrifuged briefly. 22.5 μL single reaction mix was transferred into MicroAmp™Optical-96-Well-Reaction-Plate (AppliedBiosystems®) to which 2.5 μL template gDNA was added to reach total volume of 25 μL per one reaction. Each PCR reaction plate included 8 standard dilutions, one negative reverse transcriptase, one non-template control containing ddH₂O and duplicates or triplicates of each experimental DNA template.

Standard used for PCR had 5×10^6 (5E6) virus copies (obtained from Virology Department, Umeå University Hospital), followed by 1/10 serial dilutions to reach concentrations of 5E5, 5E4, 5E3, 5E2, 5E1,

5 and 1 virus copy.

Real-time cyler thermal condition was as follow: heating (50 °C/2 min), HotStarTaq® DNA Polymerase initial activation (95 °C/10 min), denaturation (94 °C/15sec), annealing and extension (60 °C/60sec). Repeated 45 times for approximately 90 minutes. Data were analyzed using ABI-7900HT-SDS-Plate-Utility Version-2.4 software.

Results

Clinical, demographic and conventional laboratory findings for patients with positive CSF HHV-6 DNA

All patients with clinical data (100%; 7/7; {6 cases out of total 13 had missing clinical data}) presented with fever ($\geq 37^{\circ}\text{C}$) and vomiting and 6 (86%) with seizures. Male to female ratio was 1:1. Half patients were toddlers, 42% (5/12; {1 missing case}) infants and 8% (1/12) a 15 years old. Most patients (83%; 10/12) admitted to the hospital in the dry season (December to June) and 17% (2/12) in the rainy season (July to August). CSFs from 42% (5/12) were traumatic; cytological and chemical analyses were not performed. Remaining 58% (7/12) were clear; 33% (1/7) showed increased CSF WBCs (50cells/mm³) with 60% lymphocytosis, remaining 67% (6/7) showed normal count (< 5cells/mm³). CSF glucose levels for most specimens (86%; 6/7) were normal (45–100 mg/dl) and one specimen (14%) was low (30 mg/dl). CSF proteins level was high (> 45 mg/dl) in 43% (3/7), normal (14–45 mg/dl) in 43% (3/7) and low (11 mg/dl) in 14% (1/7) (Table 1). All 13 HHV-6 positive cases did not show evidence of rapid-growing-bacteria co-existing in CSF on Gram's stain and *in vitro* bacterial culture. However, 23% (3/13) were positive for other non-cultivable microbes. All patients (100%; 7/7) were recovered and discharged.

Table 1
Demographic, Clinical and Conventional Laboratory Data for HHV-6 Positive Patients

#	Demographic Data (n = 12) ⁽¹⁾		Number of Cases (% Out of 12 ⁽¹⁾)	% Out of 503 ⁽¹⁾
1	Sex	Males	6 (50%)	1.2%
		Females	6 (50%)	1.2%
2	Age	≤ 12 Months	5 (42%)	1%
		1 to 5 Years	6 (50%)	1.2%
		≥ 10 Years	1 (8%)	0.2%
3	Season of Admission	Winter (November-February)	3 (25%)	0.6%
		Summer (Mars-June)	7 (58%)	1.4%
		Autumn (July-August)	2 (17%)	0.4%
#	Signs & Symptoms (n = 7) ⁽²⁾		Number of Cases (% Out of 7 ⁽²⁾)	
1	Fever		7 (100%)	
2	Seizures		6 (86%)	
3	Vomiting		7 (100%)	
4	Chills		1 (14%)	
5	Stiff neck		1 (14%)	
#	Conventional Lab Results (n = 12) ⁽¹⁾		Number of Cases [% Out of 12 ⁽¹⁾] (% Out of 7 ⁽³⁾)	
1	CSF WBC Count	Normal (< 5cells/mm3)	6 [50%] (86%)	
		Increased (= 50 cells/mm3)	1 [8%] (14%)	
2	CSF Glucose Level	Normal (45– 100 mg/dl)	6 [50%] (86%)	
		Decreased (≤30 mg/dl)	1 [8%] (14%)	
3	CSF Protein Level	Normal (14– 45 mg/dl)	3 [25%] (43%)	
		Increased (≥ 45 mg/dl)	3 [25%] (43%)	
		Decreased (≤11 mg/dl)	1 [8%] (14%)	
⁽¹⁾ Demographic and conventional laboratory data are available for 12 (92%) out of total 13 HHV-6 positive cases				

#	Demographic Data ($n = 12$) ⁽¹⁾	Number of Cases (% Out of 12 ⁽¹⁾)	% Out of 503 ⁽¹⁾
⁽²⁾ Clinical data is available for 7 (54%) out of total 13 HHV-6 positive cases			
⁽³⁾ Five (42%) out of the 12 CSF specimens were traumatic therefore cell count, glucose and protein concentrations were not estimated			

Table 2
Ct and Virus Load for HHV-6 Positive
Individual CSF Specimens Using
Quantitative TaqMan Real-Time PCR

Sample ID	Ct	Virus Quantity Per PCR run
1	38.03	32×10^3
2	39.37	1×10^2
3	40.83	30
4	37.31	527
5	40.13	54
6	38.38	221
7	38.09	281
8	34.99	3.5×10^3
9	37.64	69
10	36.77	818
11	40.59	37
12	35.65	2×10^3
13	31.87	44×10^3

Real-time PCR findings

13/503 (2.6%) CSF specimens were positive for HHV-6 DNA by PCR. Median Ct for all positive specimens was 38 with range 31.9 to 40.8. Median virus copy was 281.3/PCR run (1×10^5 virus copies/ml CSF) with range 30 to 44×10^3 copies/PCR run (12×10^3 and 18×10^6 virus copies/ml CSF). Individual PCR data for

all 13 positive specimens are shown in table (2). Standard dilutions with viral copies of 5E6 to 5E2 produced amplification curves between Ct 24 to Ct 36. The generated standard curve plot showed repeated perfect negative association between Ct and viral quantity in all PCR runs.

Final cases classification based on clinical and molecular findings is shown on table (3).

Table 3

Classification of Cases Based on Clinical and Molecular Findings

#	Defined Groups	Cases out of 503 ⁽¹⁾		% Out of 40 ⁽²⁾
		%	Frequency	
1	Proven infectious meningitis ⁽³⁾	8%	40 ⁽²⁾	100%
2	Proven viral meningitis	3.2%	16 ⁽⁴⁾	40%
3	HHV-6 meningitis	2.6%	13	33%
<i>(1) Total number of febrile suspected patients who attended the hospital during the study period and were subjected to LP</i>				
<i>(2) 17 cases with positive microbial origin -but with normal cellular count- along with all 23 cases with CSF pleocytosis with or without positive microbial etiology</i>				
<i>(3) This information has been shared in our previous publication ²⁰</i>				
<i>(4) 13 cases with confirmed HHV-6 DNA and 3 cases with other confirmed viral nucleic acids</i>				

Discussion

HHV-6 is becoming increasingly recognized as emerging CNS pathogen, nevertheless, it has never been investigated in Sudan and very little is known in sub-Saharan Africa and the meningitis belt. In this study, HHV-6 DNA was identified in CSF of 2.6% of pediatric patients with suspected meningitis. Close findings of 1.8% (27/1,482) and 1.5% (1/65) were reported from New York, USA [21] and Southern Iran [24], respectively.

Another study in New York [11] revealed significantly higher prevalence of 40% (14/35) in patients with CNS infections who tested negative for other CNS pathogens. Among well-defined groups in our study, the prevalence was also high accounting for 33% out of 40 cases with proven infectious meningitis (Table 3). Unlike Yao [11] approach, we did not exclude cases with other detected CNS pathogens because of possible co-infections, as frequently reported [21, 25–27]. In fact, we were able to identify mixed microbial infections in three cases.

Primary HHV-6 infection almost invariably occurs in the first 2 years of life [5, 28, 29], but rare cases of CNS infections, presumed due to HHV-6 reactivation, have been reported in immunocompetent older

children and adults [22]. Among 24 HHV-6 positive patients in Tavakoli study [21], 42% were infants $\leq$ 3 years and 12.5% were teenagers $\leq$ 17 years. Out of our 13 HHV-6 positive cases; 92% were infants $\leq$ 2.3 years and 8% was a 15 years old. The ratio of males to females was 1:1 which agrees with Tavakoli's findings of 1.3:1.1.

Clinical signs and symptoms in HHV-6 meningitis are not specific [30]. Tavakoli [21] reported fever in 71%, altered mental status in 67%, headache in 29% and seizures in 33%. Other reported symptoms were muscle weakness, muscle pain and stiff neck; which are general symptoms for meningitis or encephalitis. We report fever and vomiting in 100% of HHV-6 positive cases, seizures in 86%, chills in 14.3% and stiff neck in 14.3%. None of our patients developed skin rash being the only specific -but rare- symptom in HHV-6 meningitis [30]; other studies [21, 24] concur.

Normal CSF glucose with normal or elevated proteins is the usual finding in viral CNS infections [17], as found in this study. Unfortunately, chemical analysis of the CSF is ineffective in case of viral infections; however, its significance is in distinguishing bacterial from aseptic aetiologies which is crucial preliminary step in deciding adequate treatment. CSF leukocytosis was seen in 33%. Increased numbers of CSF leukocytes ($\geq 5\text{cell/mm}^3$) indicates inflammation, however, normal cellular counts do not rule out viral aetiologies. In fact, normal CSF cellular counts in patients with proven CNS infections were frequently reported [17–20]. Normal CSF profile was reported in 25% of HHV-6 positive cases by Tavakoli [21], accordingly, HHV-6 testing should not be limited to patients with abnormal CSF profiles.

Thermal cycle (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold (exceed background level). Median Ct for our positive cases was 38 (range: 31.9–40.8). Tavakoli reported Ct values range 25.03 to 39.92. In quantitative real-time PCR, Ct values inversely correlate with viral loads, therefore, low Ct value indicates high viral load and *vice versa*. Our Ct values and viral loads were found to be significantly ($p = 0.029$) inversely correlated (-0.6 , 95% CI: -0.9 to -0.1) indicating significant variation of viral loads among our patients. Substantial variation among viral loads in patients was also reported [21].

The phenomenon of HHV-6 chromosomal integration is in debate; while some [11–13] consider it an easily identifiable condition based on the presence or absence of nucleus containing blood cells in different body compartments, Ward [16] believes the few leukocytes that are usually present in normal CSF can reveal positive viral DNA in case of HHV-6 integration. In here, CSF was clear with no observed cells in most cases (86%), while single case (14%) showed increased CSF leukocytes. Ward [16] elaborated that in order to identify a condition as chromosomal integration; viral load should be high while low virus copies would indicate infection. The average concentration of CSF HHV-6 DNA in 9 children with primary infection ($2.4 \log_{10}$ copies/ml) was significantly lower than that of 21 patients with viral chromosomal integration ($4.0 \log_{10}$ copies/ml) in Ward study. In ours, the median CSF virus concentration was 1×10^5 copies/ml with minimum and maximum virus concentrations of 12×10^3 and 18×10^6 copies/ml. While Ward [16] recommended identifying low virus copies ($\leq 10^3$) as an acute HHV-6 infection and high virus copies ($\geq 10^4$) as chromosomal integration, Collot [31] identified viral

integration in approximate concentrations of 10^3 to 10^6 copies/ml. We identified viral concentrations as low as 10^4 and as high as 10^7 . Others [11, 21] reported high CSF viral loads in patients with HHV-6 CNS infections. Some authors [12, 13] insist that chromosomal integration is a rare condition. Accordingly, we assume the detected HHV-6 DNA in our mostly cell-free CSF specimens is more likely to be from free replicating virus than from chromosomally integrated virus.

Febrile seizures appear to be caused by primary HHV-6 infection in infants with incidence of 13% in the United States [4]. Knowing that febrile seizures and vomiting were dominant symptoms among our population and the most frequent age group was children ≤ 2.3 years, therefore further supporting our assumption. Despite this, and for the sake of scientific relevance, we are not ruling out the possibility of integration among our identified cases. For this reason, studies to identify the prevalence of HHV-6 integration among healthy Sudanese population are warranted.

Conclusion And Limitation

We conclude that HHV-6 CNS infection is frequent in this population (*i.e.* identified in one third of cases with proven infectious meningitis). A major limitation in this study is that we were unable to further genotype our identified HHV-6 DNA copies because of limited CSF volume (*i.e.* all available CSF was consumed in repeated testing and identifications).

Declarations

Ethics approval and consent to participate

The ethical clearance for conducting this study was obtained from the Ethical Committee Board of Al-Neelain University. Permission to collect data and specimens was granted from hospital authorities. Patients were not contacted directly; data were obtained from hospital files and were kept anonymous at all stages of the study. Specimens were obtained from the hospital main laboratory after all requested tests were applied.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study will be available from the corresponding author on reasonable request once all related articles are published.

Competing interests

We, the authors, declare that we have no competing interests with respect to the research, authorship and/or publication of this article.

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Authors' contributions

NA developed research questions and design, collected and managed all data, performed all laboratory work, statistical analysis and interpretation, wrote and edited the text. NM advised on the approach and methodology and guided and supervised the molecular laboratory work. ME and CA contributed on the approach and methodology, supervised the molecular laboratory work, edited and proofread the manuscript and were major contributors in the overall study. IF supervised the research process throughout; contributed in the development of research questions, design and methodology, managed all logistics and clinic based activities. All authors have read and approved the final manuscript.

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