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Detection of Cytomegalovirus, Epstein-Barr Virus and Human Herpes Virus 6 and 7 DNA in Febrile Children with Cancer

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ABSTRACT

Background-Patients with cancer have long periods of severe neutropenia due to chemotherapy and are particularly vulnerable to infectious complications. Most fever episodes are treated with empiric antibacterial therapy without identifying the etiologic agent. Blood culture is the gold standard test to detect infections, but it usually presents negative results. Human herpes virus establishes latency after primary infection and the potential for complications following cytotoxic chemotherapy in the absence of allogeneic transplantation is not clearly understood. This study aimed to use molecular testing to detect EBV, HCMV, and HHV-6 and -7 viruses in serum from children with cancer. **Methods-** A Nested PCR (N-PCR) technique was used to detect viral DNA extracted from serum. All patients tested presented with fever at or above 38ºC. **Results-**The results showed that herpes virus DNA was found in 70/168 (41.6%) of the samples from pediatric oncology patients with or without neutropenia. Positive DNA for EBV occurred in 3/168 (1.8%); HCMV in 16/168 (9.5%); HHV-6 in 27/168 (16%) with liver changes in 14/27 (51%) $p<0.0049$ and HHV-7 in 24/168 (14%). The presence of two or more microorganisms was found in 9/168 samples (6%). **Conclusion-** Fever in children with cancer and herpes virus DNA was detected in some samples, but only presence of HHV-6 was associated with liver changes. The significance of these findings needs to be further evaluated to clarify their correlation with the signs, symptoms, febrile episodes and infections in children with oncology diseases.

INTRODUCTION

Fever is an important cause of morbidity and mortality in children with cancer undergoing chemotherapy and requires immediate treatment. The use of empiric broad-spectrum antibiotics is necessary, although fever of unknown origin (FUO) is reported in 80% of febrile episodes. If the fever is caused by a virus, the use of broad-spectrum antibiotics could be reduced or replaced by adequate antiviral treatment [1-3].

Primary infection with human herpes virus (HHV) is frequent in young children, establishing latency phase after primary infection in the host. HHV may be reactive in immuno-compromised patients and cause fever, pneumonitis, gastrointestinal tract disorders, hepatitis, skin rashes, and neurological syndromes [4-7].

Human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), human herpes virus 6 (HHV-6), and human herpes virus 7 (HHV-7) are very common in humans, as more than 90% of individuals over the age of 50 have antibodies against these viruses [4].

This study focused on the epidemiology of HCMV, EBV, HHV-6 and HHV-7 in children with cancer undergoing chemotherapy and presenting with fever, by identifying viral DNA in the serum using Nested-PCR (N-PCR), as well as the correlation with signs and symptoms of infection.

MATERIALS AND METHODS

A prospective cohort of children ≤ 18 years old with cancer and fever episodes, undergoing chemotherapy, on the Group in Defeat of Children with Cancer (GRENDACC) from January 2010 to November 2011were selected for the study. Blood samples of 4 mL from patients that presented febrile episodes (axillary temperature ≥38ºC). N-PCR was used to detect the presence of HCMV, EBV, HHV-6 and HHV-7 DNA. In addition, 4 mL blood and 10 mL urine samples were collected for culture tests. All patients included in this study received antibiotic treatment. No patient received HHV antiviral therapy.

Children and adolescents with an underlying malignancy with or without neutropenia were considered eligible for this study. The central nervous system (CNS), gastrointestinal system (GS), respiratory system (RS) and lungs (L) were evaluated for signs and symptoms consistent with herpes virus infection.

Collected blood was stored at -80ºC in the Virus Laboratory/FCM/UNICAMP until use. The protocol was designed in accordance with the requirements for research involving human subjects in Brazil and was approved by the Institutional Ethics Committee.

Serum DNA extraction

DNA was extracted from 200 uL of serum using the Qiagen QIAamp DNA blood mini kit (Cologne, Germany), according to the manufacturer´s guidelines. The purified DNA was eluted in 50 uL TE buffer.

Nested polymerase chain reaction (N-PCR)

The primers used and reaction conditions to perform the N-PCR were those described previously by Clinque *et al*. 1993 for EBV, Ehrnst *et al*. 1995 for HCMV, Wang *et al*. 1996 for HHV-6, and Yalcin *et al*. 1994 for HHV-7. The sizes of the nested PCR amplification products were 209, 167, 423 and 264 base pairs respectively [8-11].

RESULTS

During the study, 168 fever episodes occurred in 68 patients, with a median of 5.6 episodes per patient during chemotherapy. The patient characteristics are shown in Table I.

TABLE I. Patient characteristics

Underlying Disease	N. of patients	Sex (M/F)	Median Age	Total Fever episodes
ALL	29	14/15	6.7	70
HL	9	5/4	10.8	19
Osteosarcoma	9	3/6	14.5	21
AML	6	4/2	6.2	19
Histiocytosis	4	2/2	3.6	5
CML	3	1/2	14	3
Neuroblastoma	3	2/1	4.6	7
Biphenotypic leukemia	2	2/0	5	9
NHL	2	1/1	7.3	11
Rhabdomyosarcoma	1	1/0	3	4
Total	**68**	**35/33**	**7.0**	**168**

The median age at diagnosis was 9 years (range 0.1 to 18 years) and the average of median fever was 38.4ºC. A total of 196 blood cultures of peripheral and/or catheter blood and 130

urine cultures were collected from 68 patients using hospital protocol for fever episodes. Detection of HHV by N-PCR, blood and urine culture is shown in Table II.

Table II. Positive results of N-PCR, blood and urine culture in fever episodes of children with cancer.

Positive Patients	**N=168**	**(100%)**
Nested PCR - HHV-6	27/168	(16.1%)
Nested PCR - HHV-7	24/168	(14.0%)
Nested PCR - HCMV	16/168	(8.9%)
Nested PCR - EBV	3/168	(1.8%)
Urine culture	10/130	(7.7%)
Blood culture	8/196	(4.0%)

Positive samples for EBV, HCMV, HHV-6 and HHV-7

HHV DNA detection occurred in 70/168 (41.6%) of serum samples from pediatric cancer patients with febrile episodes.

Positive EBV DNA was detected in 3/168 (1.8%) febrile episodes. In these patients, the average of median fever was 39ºC, the total neutrophil count was $0.3 \times 10^3/mm^3$, with a median of 15.7 inpatient days, as well as presence of both EBV and HCMV DNA.

HCMV DNA was detected in 16/168 (8.9%) serum samples from patients with febrile episodes, average of median fever was 38.7ºC), total neutrophil count was $1.3 \times 10^3/mm^3$, with a median of 9.5 inpatient days.

HHV-6 DNA was detected in 27/168 (16.1%) of febrile episodes. The average of median fever was 38.5ºC; total neutrophil count was $0.9 \times 10^3/mm^3$ and 6.6 median inpatient days. In three cases more than one microorganism was detected. Patients with neutropenia that were positive for HHV-6 presented changes in liver function ($p<0,0049$) suggesting viral infection.

HHV-7 DNA was detected in 24/168 (14%) of fever episodes. The average of median fever was 38.4ºC; total neutrophil count was $0.9 \times 10^3/mm^3$, with a median of 5.1 inpatient days (Table III).

TABLE III. Patient characteristics with detection of more than one microorganism in fever episode

Disease	Fever (ºC)	Neutrophil ($\times 10^3$ /mm^3)	Clinical Evidence	Inpatient (days)	Microorganisms
ALL*	37.8	0.1	L	15	CMV. S. coag- and HHV-6
HL	38.3	0.8	RS	11	CMV and *E. coli*
AML	38.2	0.2	RS	21	CMV. *E. coli* and HHV-7
ALL	39.8	0.1	RS	15	CMV and *E. coli*
HL	39.2	0.4	RS	21	CMV and EBV
OS	38.4	0.9	RS	5	CMV and *K. pneumonia*
HL	38.3	0.2	L	11	HHV-6 and S. coag. -
ALL	38.5	0.7	-	3	HHV-7 and *E. coli* (UC)
ALL	37.9	1.8	RS	12	HHV-7 and *E. coli* (BC)
Average of Median	38.5	0.6	8/9	12.7	

Detection of more than one HHV was found in 9/168 (6%). The median of inpatient days was 12.7 (range 3-21 days), the average of median fever was 38.5ºC (range 38ºC – 39.8ºC) and clinical evidence of infection in 8/9 (89%) (Table III).

Two patients positive for HCMV by DNA detection and blood culture died; one died 11 days after hospitalization, also testing positive for *Escherichia coli* by blood culture. The other patient died after 5 days of hospitalization, testing positive for HCMV DNA and *Kleibsiella pneumonia*. Both bacterial infections presented high resistance to antibiotic therapy.

DISCUSSION

Despite considerable reductions over the past decades of infection-related mortality in patients with cancer who present FN, infections remain a major cause of morbidity and mortality in this susceptible population [12].

The treatment of fever episodes is oral and/or intravenous administration of broad spectrum antibiotics.

However, recent studies have reported a very high bacterial resistance to drugs used in hospitals and the emergence of resistant bacteria to several types of drugs consequently increases hospitalization time and the costs to support institutions [3]. In the present study, two patients showed resistance to several broad spectrum antibiotics.

Patients with hospitalization time over 6.6 days with at least one microorganism identified had a median of 1.8 × 10^3/mm^3 leukocytes and 1.0 × 10^3/mm^3 neutrophils. However, if we consider the reference values for healthy individuals, in which neutropenia is characterized by a neutrophil count under 2.1 × 10^3/mm^3 cells, the number of patients rises considerably. The hospitalization time in 145 fever episodes with absolute neutropenia, with an average hospitalization time of 9 days, while patients without absolute neutropenia, had a neutrophil count over 2.1 × 10^3/mm^3 cells, had an average hospitalization time of only 2 days (p = 0.0005). These data confirm those reported in the literature for patients with Acute Lymphoblastic Leukemia (ALL), in which 363/610 (59.5%) of infections detected in patients had a global neutrophil count > 1.0 × 10^3/mm^3 cells [13].

In the present study, positive blood culture occurred in 8/196 (4%) samples, consistent with other studies in which 52/610 (8.5%) of etiological agents were identified. Blood culture testing can identify approximately 25% of the microorganisms that cause fever [12,13]. The median diagnosis time is four days, ranging between three and six days. Detection of fungal infection was not included in this study, as Hakin *et al.* 2009 and Yee-Guardino, 2008, reported no fungal infection in a cohort of 30 children with cancer [1, 6].

Among the HHV studied, the least prevalent was EBV 3/169 (1.8%) this result confirms that the findings of Hakin, 2009 and Katsimpardi *et al* 2006, which showed less than 1% (2/610) of positivity [1, 12]. HCMV DNA was positive in 16/169 (9.5%), in concordance with Sheen *et al* 2009, in which 26/252 (10.3%) samples were positive for HCMV DNA [14,15]. In the present study, HCVM was more prevalent in cases where more than one microorganism was present 7/16 (43, 8%). HHV-6 DNA was the most prevalent HHV in this study 27/169 (16%) of patients presenting symptoms of changes in liver ($p < 0.0049$). Hubacek *et al* 2007 [16] found 107/367 (29.1%) positivity for HHV-6 and Yee-Guardino, 2008 found 7/39 (18%), using the same diagnostic technique [6]. Few studies have investigated the role of herpes virus 7 in patients with cancer and fever episodes. Persson *et al* 2003[5] analyzed 20 fever episodes for HHV-7 with no positive cases, in discordance with this study, which found 14.8% positives in febrile episodes.

Infections caused by the presence of two or more microorganisms are common and often fatal in pediatric oncology patients. Nevertheless, they have received little attention in recent studies [15]. The present study detected the presence of more than two microorganisms in 9/68 (11.8%) patients; 2/8 (18%) of these cases died. Other studies have reported a variation between 1.5% and 5.3% in mortality rates for pediatric oncology patients [16-18]. A recent study found three episodes with the detection of two or more microorganisms using blood culture and 17 infections by two or more microorganisms using real-time PCR in a total of 221 patients with febrile neutropenia [19].

The introduction of new diagnostic tests for the study of several types of microorganisms would increase the identification of etiologic agents that can cause fever [12]. In the present study, Nested PCR, enabled the identification of several HHVs 70/168 (41.6%) fever episodes, increasing etiology identification, but this research was restricted to EBV, HCMV, HHV-6, and HHV-7, and did not assess DNA quantification of these herpes virus.

Recently, quantitative real time PCR (qPCR) for EBV, HCMV, HHV-6 and HHV-7 was introduced to the Laboratory of Virus Research/FCM/Unicamp (Virus Laboratory, Faculty of Medical Sciences, University of Campinas). This technique will facilitate quantification of the viral load (copies/ml), providing an improvement in test sensitivity and a reduction in the diagnosis time, compared to blood culture and nested PCR.

CONCLUSIONS

In conclusion, this investigation does not suggest an association between the presence of EBV, HCMV and HHV-7 DNA and fever episodes in children with cancer, however, positive DNA for HHV-6 suggested infection in these patients. Further studies, including the investigation of other pathogens and DNA quantification, need to be performed in order to better understand the relationship among signs, symptoms, fever episodes, and infections in children with cancer.

The use of a viral monitoring test for pediatric oncology patients with fever can help to reduce the adverse outcomes of antibiotic therapy and improve survival when associated with signs and symptoms of clinical progression of the presumed infection.

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Ethics approval and consent to participate

Ethics approval for the study was obtained from The Ethics Research Committee, following institutions: Group in Default of Children with Cancer (GRENDACC) and Medicine College of Jundiai (FMJ), about the number CAAE 0053.0.141.141-09.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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Abbreviations	
DNA	Deoxyribonucleic acid
HHV	Human herpes virus
EBV	Epstein-Barr Virus
HCMV	Human Cytomegalovirus
HHV-6	Human Herpes virus 6
HHV-7	Human Herpes virus 7
FUO	Fever of unknown origin
NK	Natural Killer
N-PCR	Nested polymerase chain reaction
GRENDACC	Group in Defeat of Children with Cancer
FN	Fever and neutropenia
CNS	Central nervous system
GS	Gastrointestinal system
RS	Respiratory system
L	Lung
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
CML	Cronic myeloid leukemia
HL	Hodgkin´s lymphoma
NHL	Non-Hodgkin lymphoma