ORIGINAL ARTICLE

Dengue and Zika Viruses Detection in Children with Neurological Syndromes using Molecular Methods

Detecção dos vírus Dengue e Zika em crianças com síndromes neurológicas usando métodos moleculares

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Abstract

Objective. To investigate the presence of arbovirus genomes in cerebrospinal fluid (CSF) samples from children suspected of viral neurological infection, associating the results with clinical manifestations and analyzing the CSF. **Methods**. The cross-sectional study included CSF samples from 184 children and teenagers aged 1 day to 18 years old, with negative CSF microbiology tests. Molecular tests were carried out to detect arboviruses in the CSF. **Results**. Among 184 children, the genome of dengue (DENV) and zika (ZIKV) viruses were found in 44 CSF samples (23.9%) (CI 95% 17.7%-30.1%). Among those samples, 59% were positive for dengue virus, 38.6% for ZIKV, and 2.3% coinfection of DENV plus ZIKV was observed. In 67 patients with suspected infection/sepsis, 23.9% of CSF samples were positive; among 26 meningitis, 11.5% had positive results; in 22 hydrocephaly, 22.7% were positive; in 20 with convulsion, 25% had CSF positive, in 4 with encephalitis 25% were positive; in 5 intracranial hypertension, 60% were positive; 1 in 3 cases, 33.3% each, presented with neuritis and meningoencephalitis were positive; the 2 Guillain-Barré syndrome were positive. Pleocytosis occurred in 25% of positive cases, and antibiotics were used in 56.8%. We found a positivity of neurological infection by ZIKV and DENV in children even after the outbreak. Most positive samples did not show biochemical changes in the CSF. **Conclusion**. Molecular tests in CSF of children with neurological conditions contribute to the identification of the viral etiology, leading to less use of unnecessary antibiotics and reducing hospital stays.

Keywords: arboviruses; dengue; zika; children; molecular detection; central nervous system.

Resumo

Objective. Investigou-se a presença de genomas de arbovírus em amostras de líquido cefalorraquidiano (LCR) de crianças com suspeita de infecção neurológica viral, utilizando métodos moleculares e associando os resultados com manifestações clínicas neurológicas e análise do LCR. **Métodos**. Estudo transversal que incluiu amostras de LCR de 184 crianças e adolescentes com idades compreendidas entre 1 dia e 18 anos, com testes microbiológicos negativos no LCR. Foram efetuados testes para identificação de genomas de arbovírus no LCR. **Resultados**. Entre as 184 amostras de LCR, genoma dos vírus dengue (DENV) e zika (ZIKV) foram encontrados em 44/184 (23,9%) (IC 95% 17,7%-30,1%). Entre essas amostras, 26/44 (59%) foram positivas para DENV, 17/44 (38,6%) para ZIKV e, em 1/44 (2,3%), foi observada coinfecção DENV+ZIKV. De 67 pacientes com suspeita de sepses, 23,9% apresentaram positividade no LCR; entre os 26 casos de meningites, 11,5% tiveram resultados positivos; em 22 hidrocefalias, 22,7% foram positivas; em 20 com convulsão, 25% tiveram LCR positivo; em 4 com encefalite, 25% foram positivas; em 5 com hipertensão intracraniana, 60% foram positivas; em 1 em cada 3 casos, 33,3% apresentaram positividade para neurite e meningoencefalite; os 2 com síndrome de Guillain-Barré foram positivos. Pleocitose ocorreu em 25% dos casos positivos. Devido à ausência de diagnóstico, o uso de antibiótico foi realizado em 56,8% dos pacientes. Encontramos a presença de infecção neurológica pelo ZIKV e DENV em crianças em um período após o surto, enfatizando a circulação do vírus, mesmo após um período de maior incidência da doença. A maioria das amostras positivas não apresentou alterações bioquímicas marcantes no LCR. **Conclusão**. Os testes moleculares no LCR de crianças com doenças neurológicas contribuem para a identificação da etiologia viral, levando a uma menor utilização de antibióticos desnecessários e reduzindo o tempo de internamento hospitalar.

Palavras-chave: arbovírus; dengue; zika; crianças; detecção molecular; sistema nervoso central.

INTRODUCTION

Brazil is an endemic country for many arboviruses, and the most clinically relevant are the *Flaviviridae* family (DENV and ZIKV virus) and *Togaviridae* (chikungunya virus). The limitation of vaccine use, the low efficacy of antiviral treatments, and the difficult control of mosquito infestation make children a susceptible group with serious consequences[1]. These infections can be associated with many neurological disorders, from mild nonspecific to severe syndromes[2]. Shreds of evidence support the idea that the virus (DENV and ZIKV) acts directly on the CNS[2,3]. However, tests such as neuroimaging and cerebrospinal fluid (CSF) analysis are used for diagnostic confirmation. In Brazil, few studies assessed the prevalence of neurological

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manifestations related to these viruses in children[4], which have an RNA genome that allows rapid adaptation to the host and environmental conditions[5,6,7].

DENV is a serious public health problem, and neurological manifestations such as encephalopathy, encephalitis, and meningitis have been reported associated with this virus[6]. In the last 20 years, many epidemics associated with different dengue serotypes have occurred in Brazil: DENV-1 (1998), DENV-3 (2002), DENV-2 (2008), and DENV-4 (2010)[8].

Recent studies have shown that severe cases of the neurological disease caused by DENV have also moved to the younger age group, with a consequent increase in hospitalizations and deaths in this population[2,9].

In Brazil, in 2015, the epidemic of ZIKV infection was associated with an increase in cases of microcephaly in newborns and Guillain-Barré syndrome (GBS) in other patients, in addition to cases of severe neurological impairment[1,10,11].

Clinical and epidemiological management of Zika virus infection requires good diagnostic tests capable of distinguishing the Zika virus from other related viruses, as some clinical indicators do not always lead to a correct diagnosis with sufficient accuracy[11].

Although progress in this area has been incremental over decades, the recent approval of Dengvaxia for routine use represents a major advance in control and prevention efforts in 2022[12].

Climate services and health operations, including preventive personal and environmental measures, must also be implemented continuously to reduce community exposure to bites of the *Aedes spp*. mosquito vector, which is endemic in these tropical islands' rainforests[13].

The aim of this study was to show our experience in investigating cases of neurological infections caused by the arboviruses *Alphavirus, Flavivirus*, and *Bunyavirus* in CSF of the pediatric population - using molecular techniques to identify viral genomes and then to associate the results with clinical signs and symptoms. The biochemical and cytological analysis of the CSF associated with molecular identification can improve the sensitivity and specificity of the diagnosis of the infection and, thus, optimize treatment, reducing the unnecessary use of antibiotics and long hospitalization periods.

Patients and Methods

This retrospective study included patients aged from birth to 18 years old treated in the emergency care units or wards of the university hospital or maternity hospital from February 2017 to July 2018. This study was submitted and approved by the Ethics Committee for Research in Human Beings (CEP), number CAAE 59361816.3.0000.5404. A Free and Informed Consent Form was signed by patients of legal age or by the family/guardian. A lumbar puncture was performed to obtain the CSF for biochemical, cytological, and microbiological exams and molecular tests.

Inclusion and Exclusion Criteria: CSF samples were selected from patients who had one or more of the following symptoms or signs: headache, fever, seizures, focal deficit, papilledema, behavioral changes, lymphadenopathy, decreased level of consciousness, rash, arthralgia, myalgia, loss of muscle strength, respiratory or gastrointestinal symptoms or a history of exposure to the vectors, and with negative CSF results for fungi and bacteria. CSF samples with insufficient quantities for RNA extraction (<140µL) and samples obtained in repetition, trauma, or mechanical injury were excluded from the study.

Methods: aliquots of CSF were subjected to genetic material (RNA) extraction using the QIAamp Viral RNA Kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for the reverse transcription (RT) reaction in samples with extracted RNA for cDNA synthesis. Then, a polymerase chain reaction (RT-PCR) was performed to identify arbovirus genomes. To determine the Dengue virus serotypes, a semi-nested RT-PCR Multiplex protocol described by Lanciotti et al. (1992)[14] was used with some modifications.

The cDNA samples were also tested for detection of the viral genome of DENV and ZIKV by the technique of Real-Time PCR (RT-qPCR). For simultaneous detection of different Dengue virus serotypes (1-4) using the TaqMan reagent, the protocol described by Huhtamo et al. (2010)[15] was followed. For detecting ZIKV and CHIKV, the protocol described by Lanciotti et al. (2008)[16] and Lanciotti et al. (2007)[17], were considered, respectively. Nested RT-PCR reactions were performed to identify the genome of other viruses of the *Flavivirus* genus: Sant Louis Encephalitis virus (SLEV), West Nile virus (WNV), Ilhéus virus (ILHV), Yellow Fever virus (YFV), Rocio virus (ROCV)[18]; genus *Alphavirus* virus: Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV), Venezuelan Equine Encephalitis virus (VEEV), Mayaro virus (MAYV), Aura virus (AURA)[19], Chikungunya virus (CHIK)[20] and Bunyavirus genus: Oropouche virus (OROV)[21], for verification of probable cross-reaction.

Statistical analysis

Categorical variables are presented as frequency tables with absolute (n) and percentage (%) values, and descriptive statistics of numerical variables are presented as means and medians. The correlation between positivity and categorical variables was estimated using the Chi-square test and, when necessary, Fisher's exact test. The level of significance was set at 5%. Statistics were calculated using the SAS System for Windows (Statistical Analysis System), version 9.4 (SAS Institute Inc, 2002-2008, Cary, NC, USA. (Fleiss, J.L., 1981)[22].

RESULTS

In total, 184 children and adolescents (57% male) with IDH of neurological symptoms and a median age of 2.2 years (ranging from 1-18 years old) participated in the study. Table 1 summarizes the patient's characteristics and the positive results of the molecular tests for DENV and ZIKV genomes (n=44) associated with the neurological syndromes. We analyzed 184 CSF samples during the study period. Patients were studied from February 2017 to July 2018. Samples were tested using N-PCR and RT-qPCR techniques for viral RNA detection. Of the 44 biological samples that were positive for viral RNA, 26 were for dengue virus, 17 for zika virus, and 1 had both viruses.

Table 1: Patient's characteristics, positive results of the molecular tests for DENV and ZIKV genomes in CSF samples of pediatric patients with neurological manifestations and association with IDH.

Characteristics	n (total)	Total+ (%)	DENV+ (%)	ZIKV+ (%)	Coinfection DENV+ZIKV (%)
Total Samples	184	44 (23.9)	26 (59.0)	17 (39.0)	1 (2.0)
Median Age (years) (range)	2.2 (1d-18y)	1.9 (2d-18y)	1.8 (15d-17y)	2.4 (2d-18y)	- (17th)
Gender - F/M (%)	79/105	21/23 (48/52)	11/15 (42/58)	10/7 (59/41)	1/- (100/-)
IDH:		n=44 (23.9)	n=26 (14.1)	n=17 (9.2)	n=1 (0.5)
Infection/sepsis	67	16 (36.4)	12 (46.1)	4 (23.5)	0
Hydrocephalus	22	5 (11.4)	2 (7.7)	3 (17.6)	0
Convulsion	20	5 (11.4)	3 (11.5)	2 (11.7)	0
Meningitis	26	3 (6.8)	2 (7.7)	1 (5.9)	0
ICH	5	3 (6.8)	2 (7.7)	1 (5.9)	0
LLC	2	2 (4.5)	0	2 (11.7)	0
Arboviruses	2	2 (4.5)	0	1 (5.9)	1 (100)
GBS	2	2 (4.5)	0	2 (11.7)	0
Encephalitis	4	1 (2.3)	1 (3.8)	0	0
Neuritis	3	1 (2.3)	1 (3.8)	0	0
Meningoencephalitis	3	1 (2.3)	1 (3.8)	0	0
ADEM	1	1 (2.3)	1 (3.8)	0	0
DNPD	2	1 (2.3)	0	1 (5.9)	0
Facial paralysis	1	1 (2.3)	1 (3.8)	0	0
Others	24	0	0	0	0

Abbreviations: IDH, initial diagnostic hypothesis; ICH, intracranial hypertension; LLC, lowered level of consciousness; GBS, Guillain-Barré Syndrome; ADEM, acute disseminated encephalomyelitis; DNPD, delay in neuropsychomotor development; Others, epilepsy (n=7), headache (n=4), multiple sclerosis (n=3), derivation (n=2), coma (n=2), hyporesponsivenes (n=1), tetraparesis (n=1), ataxia (n=1), polyrhadiculopathy (n=1), radiculopathy (n=1), irritability (n=1).

Prevalence of DENV was 14.1% (CI 9.1%-19.1%) in CSF samples, ZIKV in 9.2% (CI 95% 5.03%-13.3%), and DENV plus ZIKV in 1 sample (0.5%). *Alphavirus* and *Bunyavirus* genus genomes were not detected;

Table 2 shows the comparison between positive and negative patients for DENV and ZIKV viruses and IDH

A total of 40/44 (91%) of patients with positive viral genome identification in the CSF had one or more signs and symptoms suggestive of systemic/neurological dysfunction. Regarding treatment, among the cases with DENV and/or ZIKV identification, 33/44 (75%) were hospitalized, 25/44 (56.8%) received antibiotic treatment (even with negative microbiological culture tests in the CSF), and 14/44 (32%) received immunosuppressive/corticosteroid therapy (Table 3).

Table 2. Comparison between positive and negative CSF for DENV, and ZIKV viruses of patients in relation to the Initial Diagnostic Hypothesis (IDH).

	Total	DENV			ZIKV		
IDH	**n**	**Pos.**	**Neg.**	**p-value**	**Pos.**	**Neg.**	**p-value**
Infection/sepsis	67	12	55	0.288	4	63	0.0001
Meningitis	26	2	24	0.045	1	25	0.0064
Hydrocephalus	22	2	20	0.734	3	19	0.2938
Convulsion	20	3	17	0.575	2	18	0.1670
ICH	5	2	3	0.595	1	4	1,000
Encephalitis	4	1	3		0	4	-
Neuritis	3	1	2		0	3	-
Meningoencephalitis	3	1	2		0	3	-
LLC	2	0	0		2	0	
GBS	2	0	0		2	0	
NPMD	2	0	0		1	1	
Others	8	2	6	0.6730	1	7	0.6730
TOTAL	184	26	158		17	147	

Abbreviations: Pos., positive; Neg., negative; ICH, intracranial hypertension; LLC, lowered level of consciousness; GBS, Guillain-Barré Syndrome; NPMD, neuropsychomotor delay. Others, acute disseminated encephalomyelitis, hyporesponsiveness, facial paralysis, tetraparesis, ataxia, polyradiculopathy, radiculopathy, irritability. More than 1 IDH was presented in any patient.

Table 3. Patients with DENV and ZIKV positive genomes, and association with the main signs and symptoms, biochemical and cytological parameters in CSF, and treatment.

Variables	**TOTAL(+) (%)**	**DENV(+) (%)**	**ZIKV(+) (%)**	**DENV+ZIKV(+) (%)**
	44 (100.0)	26 (59.0)	17 (38.6)	1 (2.3)
Signs/Symptoms:	N=44	N=26	N=17	N=1
Fever	25 (56.8)	15 (57.7)	9 (53.0)	1 (100)
Vomiting/Nausea	16 (36.4)	8 (31.0)	7 (41.0)	1 (100)
Rash	6 (13.6)	4 (15.0)	1 (6.0)	1 (100)
Irritability	14 (31.8)	6 (23.0)	7 (41.0)	1 (100)
Headache	10 (22.7)	6 (23.0)	4 (24.0)	0
Convulsion	10 (22.7)	5 (19.0)	5 (30.0)	0
ICH	3 (6.8)	2 (8.0)	1 (6.0)	0
neuromotor alteration	11 (25.0)	5 (19.0)	6 (35.0)	0
CSF Analysis:				0
Hypoproteinorrhachia (≤42mg/dl)	31 (70.0)	20 (77.0)	10 (59.0)	1 (100)
Hyperproteinorrhachia (>42mg/dl)	13 (29.5)	6 (23.0)	7 (41.0)	0
Hypoglycorrhachia	20 (45.4)	13 (50.0)	7 (41.0)	0
Leukocyte (≤3 cells/mm3)	33 (75.0)	22 (85.0)	11 (65.0)	0
Leukocyte (>3 cells/mm3)	11 (25.0)	4 (15.0)	6 (35.0)	1 (100)
Treatment:				
Hospitalization	33 (75.0)	19 (73.0)	13 (76.0)	1 (100)
ATB	25 (56.8)	14 (54.0)	10 (59.0)	1 (100)
Corticosteroids	14 (31.8)	9 (35.0)	4 (24.0)	1 (100)

Abreviattions: ICH, intracranial hypertension; CSF, cerebrospinal fluid; ATB, antibiotic.

DISCUSSION

Arboviruses are a major public health challenge due to the lack of effective antiviral treatment and difficult vector control; as a consequence, they overload health services. Children with suspected neurological diseases may present symptoms of infections in the NS; however, viral agents are not always investigated as the cause of these infections, and antibiotic treatment is carried out, even with normal CSF biochemical and cytological results.

This study highlights the great challenge of differential diagnosis of neurological manifestations in children in an endemic region for numerous types of circulating arboviruses.

It can be observed that most patients admitted to health services with some report of neurological manifestation rarely received a diagnostic hypothesis of viral infection. Molecular methods for detecting the RNA of these viruses are not included in the laboratory routine, making the differential diagnosis of infections in children's nervous system (NS) difficult[23,24].

It was verified that DENV and ZIKV viruses were present in 23.9% of the CSF surveyed samples. Several initial diagnostic hypotheses for these patients included neurological diseases. These findings, even with a lower prevalence, were also described in other studies carried out in Brazil[6,24].

We found DENV and ZIKV genomes in CSF collected in an interepidemic period (2017 and 2018) after intense circulation of DENV serotypes 1 and 2 in Campinas[25]. Several studies draw attention to the neurological complications of dengue, especially during epidemics with circulating serotypes 2 and 3[2,3]. The ZIKV epidemic in 2015-2016 in the Americas was associated with numerous reports of neurological diseases, in addition to microcephaly in newborns. There was a predominance of GBS in the regions affected by the epidemic, but cases of encephalopathy, encephalitis, meningitis, myelitis, and seizures have also been reported[25].

In 2009, the World Health Organization considered neurological manifestations as a sign of severe dengue, and there were several cases reported. A study carried out in Rio de Janeiro between 2006-2008 showed that infection caused by the DENV was responsible for approximately 50% of hospitalizations for meningoencephalitis[26].

Cases of acute disseminated encephalomyelitis (ADEM) and neuritis are reported in the literature as possible autoimmune neurological manifestations, which occur due to immunological imbalance after infection by the DENV[27].

ICH was present among the positive cases of DENV and ZIKV in our study, as recorded in Southeast Brazil in 2017, when shreds of evidence of ICH in children with meningitis were associated with DENV[28].

The biochemical parameters found in the CSF of our samples showed hyperproteinorrhachia, hypoglycorrhachia, and a slight increase in the number of leukocytes. In positive cases for ZIKV, hyperproteinorrachia was present in 41% of cases. These findings were also reported in the study by Bastos et al. (2018)[25], indicating that CSF laboratory results may show slight alterations in neurological disorders associated with viral infections.

ZIKV infection can be asymptomatic or cause mild illness; however, Guillain-Barré syndrome and other neurological complications can occur after virus infection[29]. In this study, two patients with GBS were included, and both were positive for ZIKV, confirming the previously reported findings of this association[29].

The clinical manifestations of the acute arbovirus infection in children seem to be very similar to those presented in adults; the most common signs and symptoms found may include acute fever, headache, myalgia, skin rash, arthralgia, and conjunctivitis[26,29].

According to Lima et al. (2020)[30], most GBS cases were associated with ZIKV infection in Brazil. These data show important evidence about the connection between infection by this virus and neurological diseases.

Among the cases detected by any of the viruses studied, 29.5% were less than 1 year old; these findings reinforce the potential that Congenital Zika Virus syndrome (CZS) can be present in asymptomatic newborns after delivery, highlighting the need to implement an investigation of prenatal and neonatal screening using molecular methods for ZIKV detection in endemic regions as a standardized protocol.

Oliveira et al. (2013)[28] recorded a high frequency of viral detection (62.9%) in the diagnosis of infection in the NS of children from Southeast Brazil, demonstrating the high efficiency of molecular methods such as RT-PCR for the diagnosis of these diseases. The results of this study may contribute to increasing the knowledge about the epidemiology of viral agents in pediatric NS infections. The detection of DENV in 14.1% and ZIKV in 9.1% of the analyzed CSF samples increases the relevance of this etiologic agent in NS infections. The association between the clinical features presented by the patient, and cytochemical parameters of the CSF do not seem to be sufficient for a more assertive diagnosis of infection caused by arboviruses. Therefore, molecular diagnosis is essential for the management of patients with SN infections, especially in endemic areas for DENV, ZIKV, and other arboviruses[28,29].

Pediatric patients are part of the vulnerable group for these diseases, as they have characteristics that facilitate the development of the most severe form and permanent sequelae. The identification of cases may suggest a change in the severity profile, and genomic surveillance could contribute by monitoring the possible entry of new strains of these arboviruses.

Health professionals should be aware of and consider

neurological disorders as suspected cases of arboviruses and report them to the local health department to mitigate the risk of transmission. An assertive diagnosis of CSF neuroinfection by molecular biology is extremely important, and this technique could be implemented in the laboratory routine, as many cases could receive adequate treatment, thus avoiding the unnecessary use of antibiotics and long hospitalization periods. However, more studies are needed as they are a group increasingly susceptible to viral neuroinvasion.

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