

Guillain–Barré Syndrome, Acute Disseminated Encephalomyelitis and Encephalitis Associated with Zika Virus Infection in Brazil: Detection of Viral RNA and Isolation of Virus during Late Infection

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Abstract. Zika virus (ZIKV) emerged in Brazil in 2015, which was followed by an increase of Guillain–Barré Syndrome (GBS) cases. We report the epidemiological, clinical, and laboratory findings of the first six neurological cases associated with ZIKV in Brazil seen in a reference neurology hospital in Pernambuco, Brazil. In all cases, ZIKV was detected in serum and/or cerebrospinal fluid (CSF) samples. In this case series, four cases were defined as GBS, one as acute disseminated encephalomyelitis (ADEM) and the other as encephalitis. ZIKV was detected in all cases by RT-PCR and virus isolation was successful in two patients. The time between ZIKV acute symptoms and the development of neurological manifestations varied from 3 to 13 days and ZIKV was detected between 15 and 34 days after the initial symptoms. Our results highlight the need to include ZIKV as a differential diagnosis for neurological syndromes in countries with circulation of this arbovirus. Because the viremia in these patients appears to persist longer, direct diagnostic techniques such as RT-PCR and viral isolation should be considered even if it is after the acute phase of viral infection.

INTRODUCTION

Zika virus (ZIKV) is an arbovirus of the family *Flaviviridae*, with various subtypes from two major lineages: Asian and African. *Aedes aegypti* and *Aedes albopictus* are the main vectors involved in the transmission of the virus, but the virus can also be spread by sexual intercourse^{1,2} and blood transfusion.³ Furthermore, there is by now strong scientific evidence of transmission from the mother to the fetus during pregnancy leading to microcephaly and other congenital anomalies.^{4,5}

ZIKV was first isolated in 1947 and, for many years, the disease seemed to have little importance because cases only occurred sporadically, producing a mild, febrile illness.⁶ From 2007 onwards, the first outbreaks outside Africa occurred on Yap Island, Micronesia, in the Pacific Ocean. In 2013, more than 30,000 cases were registered in French Polynesia, an epidemic that affected approximately 11% of its entire population.⁷

In symptomatic patients, the most frequent manifestations of ZIKV infection are maculopapular exanthema, low-grade fever, arthralgia, myalgia, headache, and nonpurulent conjunctival hyperemia. In 2013–2014, an increased occurrence of neurological cases, especially of Guillain–Barré Syndrome (GBS), was reported in French Polynesia after the ZIKV epidemics.^{7,8} Later, in 2016, a case-control study revealed that the cases of GBS that occurred in French Polynesia were strongly associated with previous ZIKV infection, as determined by serological tests.⁹

In Brazil, the first outbreaks of the disease were reported in November 2014, especially in the states of Pernambuco and Rio Grande do Norte.¹⁰ In March 2015, patients with clinical symptoms compatible with ZIKV infection and presenting neurological manifestations were seen at the Neurology Service of the Hospital of Restoration, in Pernambuco. Further

laboratory tests confirmed the presence of ZIKV in serum and cerebrospinal fluid (CSF) and the cases were reported to the Pan American Health Organization (PAHO).¹⁰ In 2016, other neurologic cases with ZIKV-RNA detection were reported.^{11,12}

The aim of this study is to report the first six cases of neurological manifestations associated with ZIKV in Pernambuco State during the epidemic in the Northeast of Brazil in 2015. In all cases, ZIKV infection was confirmed by RT-PCR and/or viral isolation in biological samples.

MATERIALS AND METHODS

Population, study settings, and ethical issues. We report a series of six cases of neurological manifestations attended between December 15, 2014, and June 30, 2015, in the emergency and general wards of the Neurology Service of the Hospital of Restoration, Pernambuco, Brazil. All patients had a history of clinical complaints compatible with an arboviral illness before the development of neurological disease. All subjects underwent clinical and neurological examinations. When indicated, complementary laboratory tests and imaging examinations were also performed.

All procedures were in accordance with the ethical standards of the Fiocruz Pernambuco Research Ethics Committee and with the Helsinki Declaration. All patients signed an informed consent form, except one child whose mother signed it. Table 1 summarizes patient information.

Sample collection and processing. Serum and CSF samples were collected and sent to the Department of Virology at Fiocruz Pernambuco under refrigeration and stored at –80°C until testing. Molecular diagnostic assays, virus isolation, and serology for ZIKV and dengue virus (DENV) were carried out.

Extraction of viral RNA. The QIAamp Viral RNA kit (QIAGEN, Valencia, CA) was used for the extraction of viral RNA from the samples of suspected cases of ZIKV infection. To this end, 140 µL of biological sample was used for the extraction according to manufacturer's instructions; the RNA was eluted in a final volume of 60 µL and stored in a freezer at –70°C until tested by RT-PCR.

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TABLE 1
Clinical features of patients with ZIKV-associated neurological manifestations in Recife, Brazil

Patient	Age (years)	Sex*	Clinical signs and symptoms	Number of days from disease onset to neurological symptoms	Number of days from disease onset to RT-PCR detection
1	2	F	Rash and itching	13	15
2	25	M	Fever, arthralgia and joint edema, myalgia, asthenia and anorexia, gastrointestinal symptoms (nausea and vomiting), headache	5	25
3	32	M	Fever, rash, itching, headache	4	31
4	35	M	Fever, rash, itching, arthralgia and joint edema, conjunctival hyperemia, retro-orbital pain, headache with unusual pain pattern	5	34
5	48	F	Rash, itching, arthralgia and joint edema, headache	3	27
6	53	F	Fever, rash, arthralgia and joint edema, conjunctival hyperemia, asthenia and anorexia, headache	7	27

F = female; M = male; RT-PCR = reverse transcriptase polymerase chain reaction.

Molecular assays and virus isolation. Conventional RT-PCR was performed for ZIKV using previously described methods with minor modifications, in which a two-step protocol (cDNA synthesis followed by the PCR) was used instead of a one-step method.¹³ For DENV, a well-established RT-PCR assay was used.¹⁴ PCR-positive samples were submitted to viral isolation in both C6/36 and Vero cell lines. For isolation, 50 µL of serum or CSF was inoculated for 1 h at room temperature or 37°C on monolayers of C6/36 cells and/or Vero cell lines, respectively. Cells were incubated for 7 days and monitored daily for development of cytopathic effect. At 7 days after inoculation, cellular supernatant was harvested and ZIKV infection was confirmed by RT-PCR.

Serologic assays. DENV IgM and IgG antibodies were detected using the kits Dengue IgM-capture ELISA and Dengue IgG capture ELISA (PANBIO, Inverness Medical Innovations Australia Pty Ltd, East Brisbane, Queensland, Australia), following the manufacturer's guidelines. ZIKV IgM antibodies were detected by IgM antibody capture ELISA using the Centers for Disease Control and Prevention protocol.¹⁵ Standard protocol of the Neurology Service for screening neurologic cases was performed in a private laboratory and included serology and PCR for herpes simplex virus-1 (HSV-1), cytomegalovirus (CMV), Epstein Barr virus (EBV), Human T lymphotropic virus (HTLV), Human Immunodeficiency Virus (HIV), and *Borrelia burgdorferi*.

RESULTS

This case series describe the first six neurological cases associated with ZIKV in Brazil, which were seen in a reference neurology hospital in Pernambuco, Brazil. Patients' age ranged from 2 to 53 years (median 33.5 years) and four of the

six cases were male. Most patients were from the Pernambuco capital (Recife). None of them reported travel during the 15 days before the onset of manifestation of clinical symptoms, indicating autochthonous infection. Two patients reported previous history of dengue (patients #3 and 6). One patient (patient #1) presented a history of vaccination against influenza 2 months before the manifestation of the first symptoms (Table 1). Regarding the order of appearance of symptoms, fever and skin rash were the first manifestations. Neurological involvement was detected from 3 to 13 days (median 5 days) after the initial symptoms (Table 1).

ZIKV RNA was detected from 15 to 34 days after the disease onset (median 27 days, Table 1). The RT-PCR for ZIKV RNA was positive in the serum of the five adults and in the CSF of the child with encephalitis. Isolation of ZIKV was successful in the serum from one patient with acute disseminated encephalomyelitis (ADEM) and in one patient with GBS. RT-PCR for DENV RNA were negative in all six cases. PCR and serology were also carried out in a private laboratory as part of the hospital routine for HSV, CMV, EBV, HTLV, HIV, and Lyme disease. All patients were negative for these diseases (data not shown). DENV IgM and IgG antibodies were detected in three and five patients, respectively, whereas ZIKV IgM was detected in two patients (Table 2).

Cases' summary. *Patient (1).* Patient (1) was a 2-year-old female child with history of low-grade fever, widespread rash, pruritus, and intermittent vomiting. Thirteen days after the onset of symptoms, the patient had alteration of consciousness level and content, focal convulsive crisis, motor deficit in the four limbs, evolving into coma, and requiring mechanical ventilation. Magnetic resonance imaging (MRI) resulted in signal change in high convexity, opercular, insular, talam, and

TABLE 2
Virological findings of patients with ZIKV-associated neurological manifestations in Recife, Brazil

Patient	Biological sample	DENV IgM	DENV IgG	ZIKV IgM	DENV RT-PCR	ZIKV RT-PCR	Virus isolation
1	CSF	Neg.	Neg.	Neg.	Neg.	Pos.	None
2	Serum	Neg.	Pos.	Inc.	Neg.	Pos.	None
3	Serum	Pos.	Pos.	Neg.	Neg.	Pos.	None
4	Serum	Neg.	Pos.	Inc.	Neg.	Pos.	ZIKV
5	Serum	Pos.	Pos.	Pos.	Neg.	Pos.	None
6	Serum	Pos.	Pos.	Pos.	Neg.	Pos.	ZIKV

DENV = dengue virus; inc. = inconclusive; neg. = negative; Pos = positive; ZIKV = Zika virus.

subthalammal nuclei. Patient was diagnosed with encephalitis. Current neurological assessment shows alert child in mutism, poor understanding and complete dependency, requiring constant wheelchair use.

Patient (2). Patient (2) was a 25-year-old male with complaints of headache, nausea, vomiting, and anorexia. After 5 days, he developed a progressive upward force deficit and left facial paralysis. Analysis of CSF showed albumin-cytologic dissociation. The patient was diagnosed with GBS. The patient has showed progressive clinical improvement with physiotherapy support but still remains with lower limb weakness.

Patient (3). Patient (3) was a 32-year-old male with complaints of fever, headache, and presence of rash on face, trunk and upper limbs, and pruritus. After 4 days, the patient had an upward motor deficit, associated with left facial paralysis. Analysis of CSF showed albumin-cytologic dissociation. The patient was diagnosed with GBS. Upon discharge, the patient initiated motor physiotherapy but still presents motor deficiency in the four limbs, without ambulation.

Patient (4). Patient (4) was a 35-year-old male with intermittent low-grade fever, headache, accompanied by pruritic rash on the face, trunk and upper limbs, nonpurulent conjunctival hyperemia, and atralgia and edema of slight intensity in hands and feet. After 5 days, the patient presented motor deficit in lower limbs, reaching the upper limbs in 48 hours, associated with bilateral facial paralysis. Analysis of CSF showed albumin-cytologic dissociation, and electro-neuromyography revealed involvement of peripheral and facial nerves. The patient was diagnosed with GBS. The patient completely recovered after 45 days of disease.

Patient (5). Patient (5) was a 48-year-old female with headache, absence of fever, accompanied by arthralgia and mild edema in hands, ankles, and feet. The patient reported itchy rash all over the body. On the third day of the onset of symptoms, she presented motor deficit and ascending sensory deficit. Analysis of CSF showed albumin-cytologic dissociation and electro-neuromyography was consistent with demyelinating polyneuropathy. The patient was diagnosed with GBS. She remained hospitalized for 52 days. Patient still presents motor deficit in the four limbs and walks with the aid of orthopedic equipment.

Patient (6). Patient (6) was a 53-year-old female with complaints of fever, rash, pruritus, asthenia, anorexia, retro-orbital

pain, conjunctival hyperemia, headache, pain and mild edema in hands, knees, and feet. After regression of the initial illness, she presented alteration of the level and content of the conscience, bilateral visual deficit, and motor and sensory deficit in lower limbs. Analysis of CSF showed 7 cells/mm³ and protein content of 31 mg/dL. MRI examination of the patient showed confluent and bilateral hyperintense lesions on T2-weighted image in the periventricular white matter and in the thoracic spine. The patient was diagnosed with ADEM. Despite full recovery of the level of consciousness, she still displays lower limb weakness.

Patient treatment consisted of intravenous immunoglobulin for the four cases of GBS at a dose of 0.4 g/kg/day for 5 days, whereas administration of methylprednisolone was reserved for patients with diagnosed with ADEM. Table 3 summarizes the results from complementary tests, the final diagnosis, and outcome of patients.

DISCUSSION

This study describes the first six neurological cases associated with ZIKV after its emergence in Brazil. The cases were officially communicated to PAHO in the report of December 2015.¹⁰ Detection of ZIKV RNA and/or viral isolation from these patients at the time of neurological symptoms development unequivocally confirms the infection by ZIKV. Neurological cases associated with ZIKV have been based mainly on clinic-epidemiological criteria and on confirmation by IgM serology,^{7-9,16} which is prone to cross-reaction with other arboviruses.¹⁷

The appearance of neurological cases potentially associated with ZIKV was initially described in the French Polynesia in 2013, with 42 GBS cases occurring after the outbreak, in patients with disease clinically compatible with Zika, however, without isolation of the virus.^{7,16} In Brazil, in 2015, there was an increase of reports of cases potentially associated with the virus, and later, other seven Latin countries reported an increase in the number of GBS cases.^{10,18}

Recently, 41 neurological cases described in 2013 as GBS in the French Polynesia had serological confirmation of ZIKV infection in stored serum samples.⁹ However, a recent publication questioned the interpretation of the serologic tests performed, claiming that the results cannot be concluded as

TABLE 3

Complementary examinations, diagnosis, and outcome of patients with ZIKV-associated neurological manifestations in Recife, Brazil

Patient	Findings from liquor	MRI findings	Diagnosis	Treatment	Evolution after 3 months
1	Cel: 170/mm ³ PL: 118 mg/dL	Symmetrical bilateral cortical and thalamic involvement	Encephalitis	MTP	Motor sequelae in all four limbs and cognitive impairment
2	Cel: 4/mm ³ PL: 407 mg/dL	ND	GBS	IVIG	Motor sequelae in lower limbs
3	Cel: 3.3/mm ³ PL: 154 mg/dL	ND	GBS	IVIG	Motor sequelae in all four limbs
4	Cel: 0/mm ³ PL: 81 mg/dL	ND	GBS	IVIG	Complete resolution
5	Cel: 4.7/mm ³ PL: 61 mg/dL	ND	GBS	IVIG	Motor sequelae in all four limbs
6	Cel: 7/mm ³ PL: 31 mg/dL	Confluent and bilateral hyperintense lesions on T2-weighted image in the periventricular white matter and in the thoracic spine	ADEM (NMO-like)	MTP + IVIG	Motor sequelae in lower limbs and bilateral appearance

ADEM = acute disseminated encephalomyelitis; Cel = cellularity; GBS = Guillain-Barré Syndrome; IVIG = intravenous immunoglobulin; MRI = magnetic resonance imaging; MTP = intravenous methylprednisolone; NMO-like = similar to neuromyelitis optica; ND = not done; PL = protein levels in cerebrospinal fluid.

ZIKV because of the potential cross-reaction with DENV.¹⁷ In the 2015–2016 epidemics in Colombia, Parra et al.¹⁹ reported 66 cases of ZIKV-associated GBS, of which 17 patients tested positive by RT-PCR.

More recently, in 2016, reports of other cases of neurological syndromes such as acute myelitis¹¹ and meningoencephalitis¹² began to appear, with detection of the virus by RT-PCR in the CSF.

In this case series, the time between the Zika symptoms and the onset of neurological manifestations for the four patients with GBS ranged from 3 to 5 days. In the reports from Colombia and the French Polynesia, the average time between infection and development of neurological symptoms was 4 and 6 days,⁹ respectively.¹⁹ The short time between the clinical presentation of the viral infection and the appearance of the disease for the GBS cases may indicate a direct neuropathic effect from the virus, as well as immune-mediated mechanisms with antibody synthesis that damage the peripheral nerves and spinal roots that occurs weeks after the clinical presentation of the viral infection. These and other questions need to be clarified in future studies focusing on the immunopathogenesis of ZIKV.

A relevant finding in this case series was the detection of viral RNA by RT-PCR and viral isolation many days after the initial clinical presentation of the ZIKV infection with positive samples varying from 15 to 34 days. Late sample collection was because the neurological symptoms began many days after the onset of the acute infection. An additional reason is that the patients were initially seen in primary health care units and only later sent to the Hospital of Restoration, a neurology reference hospital, where the complete investigation to define the etiology was done.

In the French Polynesian work, diagnosis was done by serology and none of the samples were positive by RT-PCR.⁹ On the other hand, the Colombian study detected 17 positive patients by RT-PCR. In Colombia case series, viral detection by RT-PCR was most common in urine (16 of 17), followed by CSF (3 of 17) and serum (1 of 17). In agreement with our study, viral detection in GBS patients occurred at late time after the onset of the symptoms of Zika infection (median 16.5 days), and in some cases, its positivity lasted for 48 days after the disease onset.¹⁹

In the two recent reports with viral RNA detection, samples were positive at 9 and 10 days in a case of acute myelitis and meningoencephalitis, respectively.^{11,20}

In its classic form, ZIKV viremia is detected in serum by PCR and virus isolation within the first 5 days after the onset of manifestation of symptoms.¹⁶ A hypothesis for late isolation in these neurological cases is that there may have been neurotropism associated with the virus maintaining a persistent viral load in central nervous system and meninges, what justifies the onset of inflammation and damage of the nervous system, with escapes of the virus for the serum, justifying the detection for such long periods.

Persistence of long-lasting viremia and detection through molecular techniques have been shown in other fluids, such as urine and, more recently, sperm.^{11,21,22} Gourinat et al.²³ detected by RT-PCR, viral load higher on urine from six patients with Zika, that remained positive for more than 20 days in one of the patients. In sperm, the virus has shown higher viremia and a prolonged excretion. In France, researchers detected a viral load of 100 thousand times greater in the

semen than in the blood and the urine 2 weeks after the onset of symptoms.²¹ In the United Kingdom, a 68-year-old man had an isolation of the virus by RT-PCR in a sample after 62 days from an acute infection.²² The persistence of ZIKV in body fluids and organs warrants further investigation.

All patients in this case series reported symptoms of previous viral infection before the onset of neurological manifestations. This is in agreement with the previous studies in French Polynesia and Colombia, which have showed symptoms in 88% and 97% of the cases, respectively.^{9,19} Rash was the most frequent symptom in our study, present in five of the six cases, with a pattern of early onset, beginning in the first 48 hours, followed by low fever (4/6) and arthralgia and joint edema (4/6) of low intensity. Rash was also a common finding in the French Polynesian (88%) and Colombian (59%) cases. Despite some differences in the frequency of symptoms, the described findings are suggestive of Zika and diverged from the clinical patterns of other arboviruses such as dengue and chikungunya.²⁴

During the diagnosis phase of the present study, it was sought to make rational use of high-cost complementary examinations, thus respecting the pharmacoeconomic principles for the health care system. MRI was requested exclusively for cases in which the neurological manifestations indicated impairment of the central nervous system. When the neurological symptoms indicated peripheral involvement, CSF analysis was chosen as the complementary examination.

The case of ADEM, which was diagnosed through clinical and magnetic resonance data, in association with increased cellularity without protein-cytological dissociation and with increased IgG (in one case), seemed to indicate impairment of the central nervous system that had not been mentioned in other studies. Regarding the GBS cases, some aspects deserve to be highlighted. CSF changes characteristic of protein-cytological dissociation were present, as well as the neurological symptoms that form part of the classic presentation of the disease.

The description of these six neurological cases confirmed by RNA detection and/or viral isolation reinforce the association of the ZIKV neurotropism and alerts for the need of including the viral research in countries with the circulation of this arbovirus for different neurological syndromes. It also suggests that viremia may be longer in these cases and techniques such as RT-PCR, and viral isolation should be considered even after the acute phase of the viral infection.

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