AN OUTBREAK OF CHANDIPURA VIRUS ENCEPHALITIS IN THE EASTERN DISTRICTS OF GUJARAT STATE, INDIA

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Abstract. An outbreak of encephalitis with a case fatality rate of 78.3% was investigated among children in Gujarat State, India. Twenty-six cases were reported. Three patients had IgM antibodies to Chandipura virus. Virus was isolated from one patient with rhabdomyosarcoma in porcine stable cell lines and in suckling mice. Chandipura virus RNA was present in 9 of 20 acute-phase serum samples, and virus sequences from the present outbreak were closely related to prototype strain (1965) and Andhra Pradesh, India (2003) isolates. Serologic and molecular assays documented the absence of Japanese encephalitis virus, West Nile virus, dengue virus, and paramyxoviruses in clinical samples. The etiologic agent was Chandipura virus, which has become an important encephalitis-causing virus in India.

INTRODUCTION

Viral encephalitis is an important public health problem worldwide. Chandipura (CHP) virus, a rhabdovirus, is transmitted to humans by sand flies. Vesiculoviruses were isolated in 1965 in the Chandipura (Nagpur) region of India in two adult patients with febrile illness[1] during an outbreak of febrile illness caused by chikungunya and dengue viruses. Subsequently, CHP virus was isolated from a child with acute encephalitis in Raipur in central India.[2] This virus was not considered to have an epidemic potential until an outbreak of acute encephalitis in children in Andhra Pradesh, India was attributed to CHP virus in 2003.[3] During this outbreak, a case-fatality rate of more than 55% was recorded. A similar outbreak of encephalitis in children was observed in Gujarat State in western India in 2004.

In India, most epidemics of encephalitis in children have previously been associated with Japanese encephalitis (JE).[4] However, several outbreaks of encephalitis with high mortality remained undiagnosed, with the first reported as early as 1954 in Jamshedpur in central India,[5] followed by those in Nagpur in 1958, in Raipur in 1965,[6] and more recently in the Warangal District of Andhra Pradesh in 1997 and 2002.[7] These outbreaks were attributed to dengue, chikungunya virus, measles, JE, and Reye's syndrome[8] because they were clinically indistinguishable and no definitive laboratory diagnosis was made. The recent outbreak in 2004 was differentially diagnosed using serologic, molecular, and isolation methods. The results of the investigations are reported here.

MATERIALS AND METHODS

Study area. The study area in India is located between 22° and 23°N and 73° and 75°E. The cases were reported from the Vadodara and Panchmahal districts. They are situated in the eastern hilly region of Gujarat, bordering Madhya Pradesh (Figure 1). This undulating region lies at an altitude of approximately 200 meters above mean sea level. The population is mostly tribal, composed predominantly of the Bhil and Bhilala tribes. The economy is agrarian with dry cultivation of paddy, maize, and cotton. Rainfall in this district is due to the southwest monsoon between June and September. Average annual rainfall ranges between 441 mm and 1,569 mm. Premonsoon showers (80 mm) were recorded in 2004; subsequently, there was no precipitation for the next four weeks.

Patients and clinical specimens. *Case definition.* A probable case was defined as a patient with a sudden onset of high-grade fever, followed by central nervous system involvement, altered senses, convulsions, or a comatose state, singly or in combination, and negative test results for malaria and other common causes of illness. A confirmed case was defined as a patient with one or more of the following laboratory results: presence of viral RNA, IgM antibodies against CHP virus in a clinical sample, seroconversion in a convalescent sample, and virus isolation.

Clinical specimens along with a detailed clinical history were collected from 20 patients for etiologic diagnosis. The samples included 20 blood or serum samples, 14 throat swabs, 8 cerebrospinal fluid (CSF) samples, and 10 urine samples. In addition, 119 blood samples were collected from apparently healthy persons in the community. Of these, 75 were from children (19 having a history of fever in the preceding month and 56 without such a history). Thirty-nine of these children resided in villages that had an encephalitis case and 36 resided in unaffected neighboring villages. All 44 adults tested (26 recovered fever cases and 18 without a history of fever) lived in the affected villages. A second blood sample was collected from one recovered case 12 days post-onset of disease. Samples were collected after informed consent was obtained from adults or legal guardians of minors. The study was reviewed and approved by the Institutional Ethics Committee of the National Institute of Virology, which complies with the Ethical Guidelines for Biomedical Research on Human Subjects of the Indian Council of Medical Research, 2000. The state health authorities conducted routine laboratory tests to rule out bacterial infections, malaria, sickle cell anemia, and other common causes of illness.

Serologic analysis. Serum samples of patients were screened using in-house enzyme-linked immunosorbent assays (ELISAs) for the detection of IgM antibodies to JE virus,[9] West Nile virus,[10] and dengue virus.[11] Sera of patients and asymptomatic persons were tested for IgM antibodies against CHP virus by an antigen-capture ELISA developed at The National Institute of Virology (Maharashtra, India), as previously reported.[3] Briefly, IgM from patient serum was captured on wells coated with anti-human IgM. Chandipura

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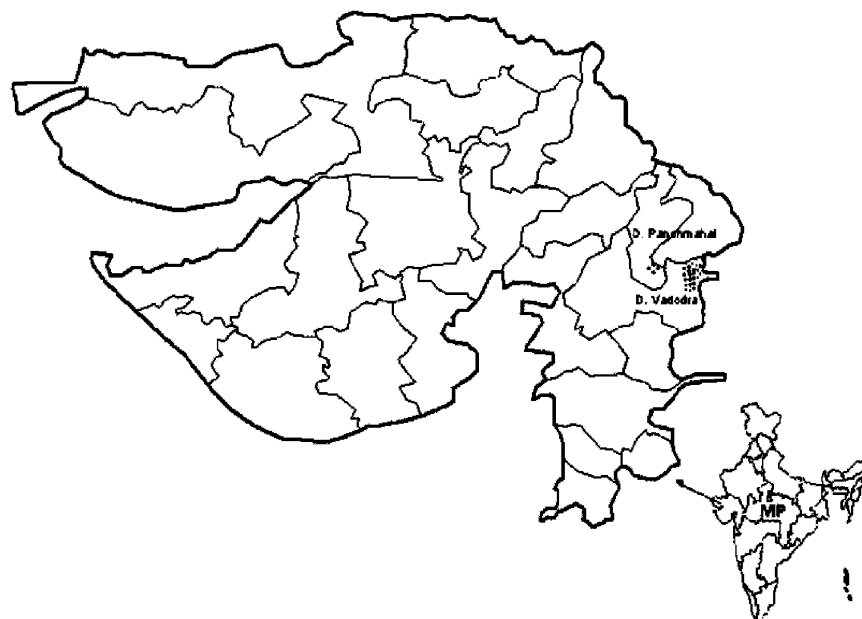
FIGURE 1. Map of Gujarat, India showing areas with cases of encephalitis. • = cases; gray lines = district borders; black lines = state borders; MP = Madhya Pradesh.

virus extracted from mouse brain by a sucrose-acetone method was the source of antigen. Captured antigen was detected with the IgG fraction of polyclonal anti-Chandipura virus mouse serum conjugated with biotin (Sigma, St. Louis, MO) and avidin-conjugated horseradish peroxidase, and *o*-phenylenediamine and hydrogen peroxide were added for color development. Negative controls included age-matched serum from apparently healthy children from an area not affected by the outbreak, and serum and CSF from children with flavivirus encephalitis. The cutoff value was determined as mean optical density for negative controls plus 3 SD.

Similarly, all sera were tested with the *in vitro* neutralization test in Vero cell cultures to detect antibodies to CHP virus.[12] Briefly, Vero cells were seeded in 96-well microtiter plates at a concentration of 3×10^4 cells/well. Aliquots of serum samples diluted 1:10 in Dulbecco's modified minimum essential medium (DMEM) with 2% fetal bovine serum (FBS) were heat-inactivated at 60°C for 20 minutes. Three-fold dilutions of serum samples starting at 1:10 were added to DMEM plus 2% FBS and mixed with an equal volume of 100 50% tissue culture infectious doses ($TCID_{50}$s) of CHP virus and incubated for one hour at 37°C. The serum-virus mixture (100 μL) was then transferred to Vero cell monolayers. The CHP virus immune mouse serum was used as a positive control and normal mouse serum was used as a negative control. Virus controls and cell controls were included in each plate and virus titration was also carried out. The virus neutralizing antibody titer was expressed the reciprocal of the highest antibody dilution capable of neutralizing 100 $TCID_{50}$s of virus. A titer of 1:10 was considered a positive result.

Detection of viral RNA. A nested reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out for the detection of flavivirus (serum), paramyxovirus (urine), and enterovirus (serum) RNA in the clinical specimens according to previously described methods.[2] The enteroviral RNA-positive PCR products were sequenced as described in this report and the homology of the sequences was compared with the sequences from the GenBank database using the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information, Bethesda, MD) (http://www.ncbi.nlm.nih.gov/Tools/). For the detection of CHP virus RNA, RNA from the clinical specimens was isolated using the Trizol LS reagent (Life Technologies, Inc., Gaithersburg, MD) according to the instructions of the manufacturer. Following preparation of cDNA using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) for one hour at 42°C, a two-step DNA amplification was carried out with 35 cycles each of denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one minute. The PCR products were subjected to electrophoresis on 2% agarose gels. The primers used were CHAND-G-F2, 425–445: 5′-GTC TTG TGG TTA TGC TTC TGT-3′; CHAND-G-R5, 750–771: 5′-TTC CGT TCC GAC CGC AAT AACT-3′; CHAND-G-F5, 541–560: 5′-GAG AAT GCG ACC AGT CTT AT-3′; and CHAND-G-R6, 724–744: 5′-TGC AAG TTC GAG ACC TTC CAT-3′. The expected size of the nested PCR product was 204 basepairs. Negative controls were included in all PCR assays. Pre-amplification and post-amplification were conducted on the different floors of the laboratory.

Sequencing and phylogenetic analysis. The PCR products were purified using Wizard PCR preps DNA purification kit (Promega) according to the manufacturer's instructions and subsequently sequenced using the Big Dye Terminator cycle sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an automatic Sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). Multiple alignments of nucleotide sequences were carried out using Clustalx version 1.8 (European Molecular Biology Laboratory, Heidelberg, Germany). Phylogenetic analysis based on the partial glycoprotein gene (G gene) sequences (203 nucleotides) was carried out using MEGA version 2.1 software.[13] For analysis in MEGA, the Jukes-Cantor distance model was used with the neighbor-joining algorithm. The reliability of different

phylogenetic groupings was evaluated by using the bootstrap test with 1,000 bootstrap replications available in MEGA. The percentage nucleotide identity (PNI) was calculated by using p-distance available in MEGA.

Isolation of the etiologic agent. Six CHP virus RNA-positive clinical samples were inoculated intracerebrally in two-day-old Swiss albino mice. The mice were observed for 14 days for signs of illness. For *in vitro* amplification of the etiologic agent, specimens from 13 patients (7 serum, 5 whole blood, 6 throat swabs, and 2 cerebrospinal fluid samples) were inoculated in rhabdomyosarcoma (RD) and porcine stable (PS) cell lines. Cultures were observed for the development of cytopathic effects (CPEs). Cultures not showing CPEs for three passages were considered negative for CHP virus.

Collection of sand flies. Adult sand flies were collected from houses and peridomestic habitats in the villages with encephalitis cases. They were transported in plastic jars and cloth cages, identified, pooled, and stored at −70°C until processed. Seventeen pools composed of 81 sand flies were subjected to RT-PCR for detection of CHP viral RNA.

RESULTS

Clinicoepidemiologic features. Twenty-six probable encephalitis cases (22 from the Vadodra district and 4 from the Panchmahal district) were reported in children between June 9 and July 14, 2004. The case distribution was rural and spread over three talukas (counties) in Vadodara and one taluka in Panchmahal (Figure 1). Clustering of cases was not observed. The male to female ratio was 1:1 and the ages of the patients ranged between 2 and 16 years (mean age = 6.03 years). Eighteen of 23 patients died, giving a case-fatality rate of 78.3% (the outcome in three patients was not known), and 13 (72.2%) of 18 died within 24 hours of onset of disease. The remaining deaths occurred 2–4 days post-onset of illness. Neurologic sequelae were not observed in survivors.

Detailed clinical findings for 19 patients were available for analysis. The most common manifestations were fever and altered senses in all patients, convulsions in 17, vomiting in 13, diarrhea in 10, chills preceding fever in 3, and cough in 3.

The nutritional status of the patients ranged from poor to average. Vesicular eruptions with serous transudate and hyperpigmentation on healing were observed in four patients. Tachycardia was observed, which was consistent with an increased temperature. Eight of 19 patients had bilateral crepitations on auscultation of the lungs. Two children had palpable livers with marginally increased levels of alanine aminotransferase and aspartate aminotransferase. Nine children had decreased muscle tone and power. Hemiparesis and seventh cranial nerve palsy were detected in one patient. Deep tendon reflexes were not elicitable in 13 patients, and were normal and lower in three patients each. The plantar reflex was elicited in 10 patients, abnormal (extensor plantar response) in 7 patients, and equivocal in 2 patients. Cracked pot sound was present in two patients.

Clinical laboratory test results. Hemoglobin levels ranged from 7 to 10.5 mg% (normal range = 10.5–15.5 mg%). Total white blood cell counts ranged from 3,600 to 12,500 cells/mm^3 (normal range = 4500–11,000 cells/mm^3). The differential white blood cell count was neutrophils = 54–80% (normal range = 54–62%), lymphocytes = 20–46% (normal range = 25–35%), eosinophils < 3% (normal < 5%), and monocytes =

1–2% (normal range = 2–8%). The erythrocyte sedimentation rate was less than 20 mm/hour (normal <1 5 mm/hour) in seven cases studied. Blood glucose levels ranged from 56 to 216 mg/dL (normal range = 60–110 mg/dL).

The CSF in 13 patients was clear and colorless, with a normal or insignificantly increased opening pressure, and a white blood cell count within normal limits (0–5 cells/mm^3). No red blood cells were present. The protein level was < 60 mg% in nine patients and ranged from 90 to 210 mg% in the remaining four patients (normal range = 15–60 mg%). The CSF glucose levels ranged from 10 to 120 mg/dL (normal = 50% of blood glucose levels), and were less than 50% of serum glucose levels in two patients. C-reactive protein was absent in all samples. No evidence of sickle celled anemia was found in any of the children examined.

Etiologic investigations. Of the 20 clinical specimens from 13 patients analyzed, one serum sample collected two days post-onset of symptoms (NIV-045983) showed CHP virus in RD and PS cell lines, as well as in infant mice. This isolate was confirmed by PCR and sequencing.

Nine (45%) of 20 acute-phase serum samples were positive for CHP virus RNA, including that of the patient who showed seroconversion to IgM antibodies to CHP virus in the second sample. Flavivirus and paramyxovirus RNA was not detected in nine serum and five urine samples, respectively. All throat swabs, urine samples, and CSF samples were negative for CHP viral RNA. Two serum samples were positive for enterovirus RNA. BLAST analysis of the two sequences showed 96% homology with echovirus 11 and human poliovirus 1 isolates, respectively.

All acute-phase sera were negative for IgM antibodies to JE, dengue, and West Nile viruses. Three patients were positive for IgM antibodies to CHP virus; 2 of 20 acute-phase samples were positive for IgM and neutralizing antibodies to CHP virus. In addition, the only patient whose convalescent sample was available on the day 12 post-onset of symptoms was positive for IgM and neutralizing antibodies to CHP virus, although the acute-phase sample was negative for both antibodies. One serum sample from a 16-year-old female was negative for IgM antibodies to CHP virus and positive for neutralizing antibodies to CHP virus. The neutralizing antibody titer was 1:90 in all patients. The remaining acute-phase sera were negative.

Serologic tests conducted in apparently healthy population showed that 10.6% of the children were positive for IgM antibodies to CHP virus and 65.3% were positive for neutralizing antibodies (geometric mean titer [GMT] = 28.17), which was significantly lower than among patients ($P < 0.01$)]. Age-wise analysis showed that children less than five years of age had a significantly low frequency of neutralizing antibodies (8 of 18, 44%) compared with those more than five years of age (28 of 38, 73.3%) ($P < 0.05$). Among adults, 4.5% were positive for IgM antibodies to CHP virus and 97.7% were positive for neutralizing antibodies (GMT = 27.6).

Phylogenetic analysis. The GenBank Accession numbers for the sequences generated during this study are AY–871791–99. The phylogenetic status of the CHP viral sequences obtained from the clinical samples and one isolate during the present outbreak is presented in Figure 2. Overall, the PNI between different CHP viral isolates ranged from 91.1% to 100%. Of the three major clusters identified, six Varodara sequences formed one cluster; three Andhra
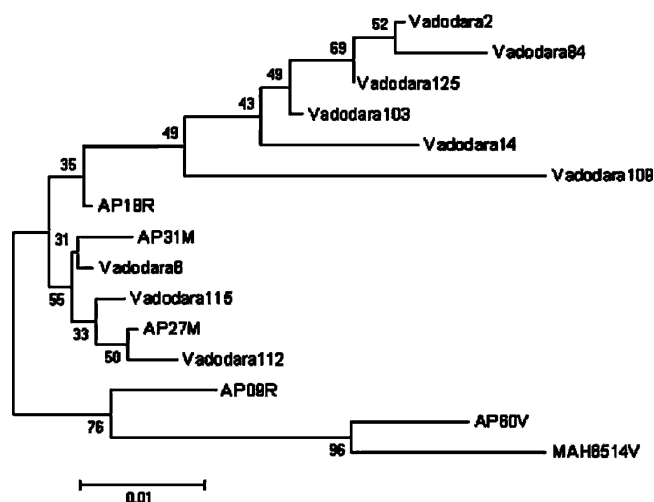
FIGURE 2. Phylogenetic analysis of Chandipura viruses based on partial G gene sequences (203 nucleotides). Percent bootstrap support is indicated by the values at each node. The accession numbers for the Chandipura virus G gene sequences used for analysis include five sequences from the outbreak in the state of Andhra Pradesh (AP) in 2003: AP18R (AY 614722), AP31M (AY 614721), AP27M (AY382603), AP09R (AY 614723), AP60V (AY 614720); one sequence from the state of Maharashtra in 1965: MAH6514V (AY614717); and nine sequences obtained during this study in 2004.

Pradesh and three Varodara sequences formed a second cluster, and two Andhra Pradesh sequences and the prototype 1965 strain isolated from Maharashtra State formed the third cluster. The PNI between groups was 96.7% (range = 95–98.5% for clusters 1 and 2), 93.8% (91.1–96.6% for clusters 1 and 3), and 95.9% (range = 94.4–97.8% for clusters 1 and 2). Thus, all sequences were closely related to each other and an epidemic was not characterized by circulation of only one type of isolate.

Sand fly species composition and RT-PCR results. A total of 27 female and 54 male *Sergentomyia* sand flies collected between July 9 and 11, 2004 from peridomestic habitats were processed in 17 pools. The RT-PCR conducted on all pools did not show any positive results.

DISCUSSION

Demonstration of CHP viral RNA in 9 (45%) of 20 cases, IgM antibodies to CHP virus in 3 (15%) of 20 cases, and 1 isolation of CHP virus is strong evidence for CHP virus as the etiologic agent of the encephalitis outbreak that affected children in tribal areas in Gujarat state, India in June–July 2004. The absence of neutralizing antibodies to CHP virus in most (17 of 20) of the patients indicates a primary infection with CHP virus. One patient, a 16-year-old girl, had neutralizing antibodies to CHP virus in the absence of IgM antibodies to this virus in an acute-phase serum sample. This is indicative of past exposure to CHP virus and the present episode may represent encephalitis due to an agent other than CHP virus. Whether or not the detection of enteroviral RNA in two patients without CHP viral markers is incidental or represents enteroviral etiology cannot be ascertained.

The results confirm the limitations of the ELISA and usefulness of molecular assays in the diagnosis of CHP viral encephalitis with a rapid clinical course and high mortality. Since most (13 of 18, 72.2%) of the patients died within 24 hours, IgM antibodies could be demonstrated only in 2 (10%) of 20 patients. Importantly, one surviving patient who pro-

vided a blood sample on the 12th day post-onset of disease was negative for IgM antibodies to CHP virus. The acute-phase sample from this patient was negative for IgM antibodies to CHP virus but positive for CHP viral RNA. Lack of molecular/serologic evidence of CHP virus infection in 9 of 20 cases may represent the absence of viremia at the time of sampling and/or an etiology not related to CHP virus. The dynamics of viral RNA and antibodies in patients with CHP viral encephalitis needs to be determined for designing appropriate diagnostic methods.

Phylogenetic analysis based on conserved G gene regions placed six Vadodara sequences in a distinct cluster and three sequences in another cluster along with the AP isolates of 2003, suggesting co-circulation of closely related CHP viruses in the same epidemic.

The clinical picture was consistent with that described in an earlier outbreak.[3] The mortality rate during the present episode was higher (18 of 23, 78.3%) in comparison with that in Andhra Pradesh epidemic in 2003 (183 of 329, 55%). This could be attributed to the poor nutritional status, lack of proper management, the small scale of the outbreak.

Blood samples taken from healthy individuals showed that more than 10% of children (with or without a history of fever in the preceding month) had IgM antibodies to CHP virus, indicating recent exposure to the virus and milder forms of the disease, either sub-clinical or as self- resolving pyrexia. A total of 4.5% of the adults had IgM antibodies to CHP virus in their blood, which also indicated recent infection, although no encephalitis cases were observed among adults. Our finding of a significantly lower frequency of neutralizing antibodies among younger children suggests that a significant proportion of the pediatric population is susceptible to CHP virus and is consistent with the fact that encephalitis cases occurred only among children. Infections with CHP virus seem to be widespread in this region, as shown by the high frequency of neutralizing antibodies among the healthy population and the fact that immunity increases with age, which is suggestive of this area being endemic for CHP virus. Gujarat is the fourth

state in India to report CHP virus; the others are Maharashtra, Madhya Pradesh, and Andhra Pradesh.

Sand flies have been implicated as vectors of CHP virus. However, in our study, we could did detect CHP viral RNA in the limited samples processed. Previously, CHP virus was isolated from sand flies during the outbreak in Andhra Pradesh,[14] as well as in Maharashtra.[15]

In conclusion, CHP virus has emerged as an important encephalitis-causing pathogen in India. We emphasize the need for immediate in-depth analysis of the transmission mechanisms of this virus, including the role of vectors and other ecologic factors, for the development of appropriate control strategies.

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