Original Article

Trend of Japanese encephalitis in North India: evidence from thirty-eight acute encephalitis cases and appraisal of niceties

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Abstract

Background: In the year 2005, an epidemic of Japanese encephalitis (JE) occurred in the northern states of India. The present study was planned to reconfirm the circulation of JE in the area and to assess the trend of the disease to slow down the burden of JE.

Methodology: Surveillance was conducted to identify patients with acute encephalitis. Blood and cerebrospinal fluid specimens_ from suspected cases underwent pathological, serological, and demographic investigations. Viral testing for evidence of Japanese encephalitis virus (JEV) infection was also performed, either by IgM capture ELISA/RT-PCR or both. To identify circulating JEV strains, RT-PCR, sequencing and phylogenetic analysis was performed. Based on clinical cases reported between 1992 and 2008, the trend of JE infection in the state was analyzed to examine the dynamics of infection.

Results: Our investigations (n = 38) revealed that only 55.3% cases were positive for JE. Pathological examination revealed marked pleocytosis in CSF (90 ± 76.9 cells/mm^3), and peripheral leucocytosis (64.7 ± 8.86% neutrophils) with mild anemia. Males were more susceptible than females with a ratio of 1.63:1 and significant gender difference ($P < 0.05$) was observed in patients below six years. In the patient group younger than six years, the rate of infection per million was six-fold higher ($P < 0.005$) in males as compared to females. Our phylogenetic study suggests that the circulating strain during the 2005 JE epidemic was close to GP78, and in the future a larger epidemic may occur.

Conclusions: The 2005 JE epidemic was possibly caused by JEV GP78 and it is spreading into newer areas. The trend of JE suggests that the problem in North India is escalating and larger epidemics may occur in the future; therefore, serious steps are necessary to combat JE, including the development of more efficient surveillance methods and differential diagnosis.

Key Words: encephalitis; Japanese encephalitis; virus; outbreak; seasonal incidence; Uttar Pradesh; India; trend

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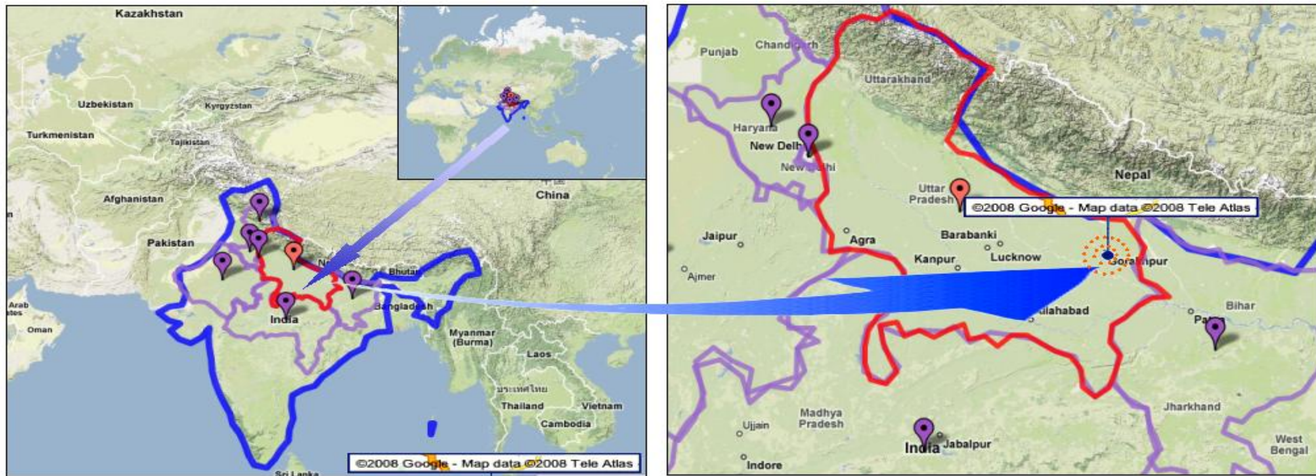
Introduction

Almost every year, so-called "undiagnosed illnesses" invade India and unfailingly claim thousands of lives. Viral encephalitis, a major global emerging public health problem, is among them. Although viruses are the most common cause of encephalitis, bacteria, fungus, and parasites may also be responsible for infection. In India, although many encephalitis outbreaks have been reported since 1955 [1], several have remained undiagnosed. In the absence of a defined cause, these outbreaks were tentatively attributed to Reye's syndrome, dengue, chikungunya, Japanese encephalitis (JE) and measles. Between July and December 2005, a large and severe epidemic of viral encephalitis was seen in northern India. The disease gripped Uttar Pradesh, border areas of Bihar and Nepal. During this interval a total of 6097 JE cases with 1,398 deaths were reported [2,3]. The case fatality rate (CFR) was 22.9%. Uttar Pradesh was most affected with 5,737 JE cases and 1,334 deaths (CFR 23.3%) and Bihar experienced 360 cases with 64 deaths (CFR 17.8%) [2]. However, the true incidence was much higher, as most of the cases remained asymptomatic and undiagnosed.

The majority of the cases during this epidemic came from eastern Uttar Pradesh (Gorakhpur and adjoining areas), which is the paddy growing "Terai area." Uttar Pradesh (a northern state of India) lies between latitudes 24° and 31° north and longitudes 77° and 84° east (Figure 1) and is surrounded by Uttaranchal in the northeast, Haryana and Himachal Pradesh in the north, Delhi and Rajasthan in the west, Madhya Pradesh in the southwest, Chhattisgarh in the south, Bihar in the southeast, and

Figure 1. Japanese encephalitis affected areas of India, 2005. The map of India shows Uttar Pradesh outlined in red, the area which was worst affected by JE in 2005 (Map modified and used with permission from Google™ Maps, CA, USA).



Nepal along the East. After the 2001 census, Uttar Pradesh is the most populous state in India, accounting for 16.4% of the total population of the country. Population density of this state is 689 persons per square kilometer, while it is 324 persons per square kilometer for the country. Children aged 0 to 6 years make up 18.35% of the population, of which 9.58% are male and 8.77% are females, while approximately 40% of the total population belongs to the 0-12 year age group. The rural population is 79.22% [4]. There are three distinct seasons: Summer (March to June, with temperatures ranging from 27.5º-32.5 ºC, with a max 45 ºC); Monsoon (July to October, with rainfall of 1,000-2,000 mm in the east, and 600-1,000 mm in the west); and Winter (November to February, with temperatures ranging from 12.5º-17.5 ºC). The entire state has a tropical monsoon climate.

JE is an acute viral zoonotic infection of the central nervous system (CNS), which produces meningomyelo-encephalitis. It poses a serious public health problem with an increasing frequency of epidemics and outbreaks in many parts of the Indian subcontinent and South East Asian countries over the last four decades. Now approaching newer areas such as Papua New Guinea and Australia, it has been classified as new emerging disease [5,6]. JE virus (JEV) is an arthropod-borne flavivirus and is transmitted to human beings by *Culex tritaeniorrhynchus* and other related rice field-breeding mosquitoes of genus *Culex*. The disease commonly affects children and is a major cause of acute childhood encephalopathy. JEV or antigenically related virus has been identified serologically in different parts of India since the mid-fifties. Although initial cases of JE were reported in the 1950s in India, Uttar Pradesh saw its first epidemic in 1978 [7]. Since then, this encephalitis has taken more than 10,000 lives in the state [8,9]. As in 2005, most of the cases of acute encephalitis came from Gorakhpur and adjoining areas. In the present study, patients with acute encephalitis were enrolled from this area and examined for the evidence of JE infection with clinicopathological, serological, and demographical features. Based on current evidence and historical epidemics of JEV in Uttar Pradesh (India), we will discuss the current trend of the disease to prepare ourselves for the possibility of a future epidemic.

Materials and Methods

Outbreak Surveillance and sample collection

After approval from the Institutional Review Board of the Centre for Cellular and Molecular Biology, Hyderabad, surveillance was conducted at Gorakhpur and adjoining areas to identify patients with acute encephalopathic illness during July to November 2005. A patient was suspected to have encephalitis if all the following criteria were met: (i) a recent history of fever; (ii) cerebrospinal fluid (CSF) protein concentration of at least 40 mg per deciliter or white blood cell (WBC) count of at least 5 per cubic millimeter; (iii) no other clinical diagnosis; (iv) neurological impairment. Mild and acute encephalitis were categorized on the basis of severity of symptoms. Mild encephalitis was characterized by mild fever and headache, and the patient testing positive for JE-ELISA. However, a

recent history of any one or more symptoms including altered sensorium, headache, stiffness of the neck, tremors, vomiting, altered mental status, seizure, myalgia, abdominal pain and depressed level of consciousness with coma and paralysis along with fever along with positive testing for JE-ELISA indicated acute encephalitis. No tests were performed for Reye's syndrome, dengue, chikungunya or any other flavivirus infections. A total of 38 patients with symptoms of acute encephalopathic illness were enrolled for this study (Table 1). After the written informed consent of the patient/patient's guardian was obtained, blood and/or CSF samples were collected and stored at –20°C. No patient had history of JE vaccination. All JE patients were examined completely for clinico-pathological symptoms as described above, and previous clinical history and demographic information about the patients were obtained.

Laboratory investigation Blood and/or CSF specimens from patients underwent serological and viral testing for evidence of arboviral infection by IgM-capture ELISA. For every examination, serum and/or CSF samples were collected by single aseptic venipuncture or lumber puncture respectively. All the serum and CSF samples were tested for the presence JEV by IgM-capture ELISA (JEV CheX IgM ELISA Kit, XCyton Diagnostics Ltd., Bangalore, India) according to the manufacturer's instructions. Briefly, CSF and serum samples were diluted 10- and 20-fold respectively and 100 µl of test CSF/serum samples with appropriate controls were incubated in ELISA plates for one hour at 37ºC. To each well 100 µl of JEV antigen was added and incubated at 37ºC for one hour and washed again. Next, 100 µl of biotinylated-monoclonal antibody was added and incubated for 30 minutes at 37ºC and 100 µl of streptavidin-peroxidase conjugate was added. The plate was kept at room temperature for 15 minutes and 100µl of substrate was added to each well and the plate was left for 10 minutes at room temperature. Finally, 100 µl of stop solution was added to each well to arrest the reaction. The plate was read at 450 nm in an ELISA reader (SpectraMax 190, Molecular Devices, Sunnyvale, California, USA) within one hour of reaction. A positive reaction of the CSF sample confirmed JEV infection and a positive reaction of the serum sample suggested a recent JEV infection. Samples were considered as JE positive if the ELISA value was more than 100 ELISA units; otherwise, samples were considered as JE negative [10] (Table 1). This kit is already evaluated for its sensitivity, specificity, and usability against JE patient samples by various workers [11,12]. If the blood and CSF samples drawn from a patient within first five days of fever were found non-reactive, a second serum/CSF sample was collected from that patient after seven days of the occurrence of the fever. Clinical investigations of JE positive patients included the following: complete peripheral blood examination; blood glucose level; CSF examination for appearance with cell count; measurement of glucose and protein; bacterial culture; and gram staining.

Molecular and Phylogenetic analysis

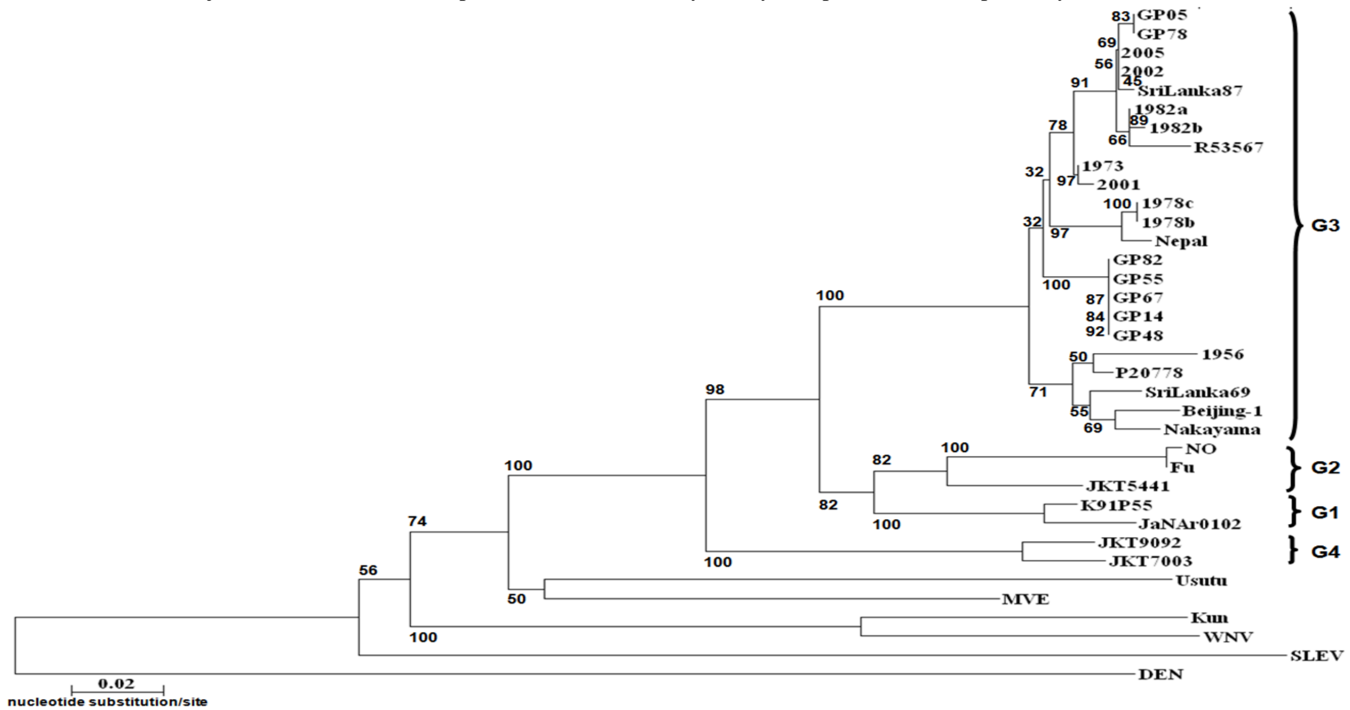
Notably, we could also extract viral RNA, specifically GP05, from a CSF sample (I.D. 17). Viral RNA was isolated using a GF-1 Viral RNA/DNA Extraction Kit (Vivantis Inc., California, USA) according to the manufacturer's instructions. The full *env* gene of GP05 was amplified by standard reverse-transcriptase polymerase chain reaction (RT-PCR). RT-PCR was performed with an RT-PCR Kit (Promega, Madison, WI, USA) using 5'CTGTTGGTCGCTCCGGCTTACAG3' as the forward primer and 5' AGCATGCACATTGGTCGCTAAGAA3' as the reverse primer. Primers were designed against the *env* gene of JEV GP78 (AF075723) as a reference sequence by using Gene Tool software (BioTools Inc., Edmonton, Canada) and synthesized by Bioserve Biotechnologies India Pvt. Ltd., India. Briefly, for a 20 µl RT-reaction, 100 mM $MgCl_2$, 10X RT Buffer, 10mM dNTPs, 0.5 unit RNasin, 2.5 unit of reverse transcriptase, 15 picomolar reverse primer and 200ng viral RNA were added. RT was performed at 47°C for one hour. Second strand synthesis was performed by PCR for a 20 µl reaction using 10X PCR Buffer (Applied Biosystems, USA), 37 mM $MgCl_2$ (Applied Biosystems, USA), 5 mM dNTP mix (Eppendorf, Germany), 2 pM each of forward and reverse primers, 2 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA), and 1µl of cDNA. Amplification was carried out for 35 cycles in Eppendorf Mastercycler EP (Eppendorf, Germany), each consisting of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds,

Table 1. Information sheet of patients (n = 38) with acute encephalitis in Uttar Pradesh (India), 2005.

I.D.	Sex	Age	Sample		JEV-IgM EU		Result
			Serum	CSF	Serum	CSF	
1	F	11Y	N	Y	N	-2.69	JE -ve
2	M	8Y	N	Y	N	3.07	JE –ve
3	F	10Y	N	Y	N	3.07	JE –ve
4	M	4Y	Y	N	699.22	N	JE +ve
5	F	11Y	N	Y	N	3.46	JE -ve
6	M	8Y	N	Y	N	-0.76	JE –ve
7	F	8Y	Y	N	61.47	N	JE –ve
8	F	6Y	N	Y	N	-4.46	JE –ve
9	M	6.3Y	N	Y	N	-2.74	JE –ve
10	M	11Y	N	Y	N	168.04	JE +ve
11	M	8Y	N	Y	N	250.51	JE +ve
12	M	5Y	N	Y	N	293.12	JE +ve
13	F	7Y	N	Y	N	275.25	JE +ve
14	F	6Y	N	Y	N	494.84	JE +ve
15	F	6Y	N	Y	N	91.75	JE –ve
16	M	10Y	N	Y	N	-0.68	JE –ve
17	F	8Y	N	Y	N	103.09	JE +ve
18	M	4.6Y	Y	N	499.59	N	JE +ve
19	F	12Y	Y	N	416.06	N	JE +ve
20	M	5Y	Y	N	6.82	N	JE –ve
21	M	7Y	Y	N	285.54	N	JE +ve
22	M	10Y	Y	N	223.92	N	JE +ve
23	M	8.6Y	Y	N	592.8	N	JE +ve
24	F	8Y	Y	N	300.34	N	JE +ve
25	M	6Y	Y	N	182.19	N	JE +ve
26	M	–	Y	N	13.7	N	JE –ve
27	F	8Y	Y	N	167.51	N	JE +ve
28	F	–	Y	N	46.19	N	JE –ve
29	M	–	Y	N	13.7	N	JE –ve
30	F	9Y	Y	N	85.88	N	JE –ve
31	M	6Y	Y	N	534.7	N	JE +ve
32	M	7Y	Y	N	171.76	N	JE +ve
33	F	8Y	Y	N	255.29	N	JE +ve
34	F	8Y	Y	N	277.19	N	JE +ve
35	M	8.6Y	Y	N	793.56	N	JE +ve
36	M	4Y	Y	N	769	N	JE +ve
37	M	7Y	Y	N	11.11	N	JE –ve
38	M	1Y	Y	N	29.23	N	JE –ve

and extension at 72°C for 2 minutes. Pre-denaturation and a further extension were performed at 95°C for 10 minutes and 72°C for 10 minutes respectively. Sequencing of the amplicon was performed on an ABI 3130 sequencer (Applied Biosystems, USA) with the BigDye Terminator cycle sequencing ready reaction kit with the primers mentioned above. Phylogenetic analysis of GP05 was performed with other known Indian as well as Southeast Asian JEV strains considering other flaviviruses as outlined. All the sequences were retrieved from NCBI (Table 2) and a neighbour-

Figure 2. Phylogeny of Japanese encephalitis virus GP05 isolated from the Gorakhpur 2005 epidemic, with reference to other Southeast Asian isolates and *flaviviruses* based on partial *env* gene sequence. The tree was generated by neighbor-joining method. Bootstrap values are indicated at the branch points. DEN, WNV, KUN, SLEV and MVE denotes Dengue virus, West Nile virus, Kunjin virus, Saint Louis encephalitis virus and Murray Valley encephalitis virus respectively.



joining tree was constructed using the ClustalX (1.83) alignments program in Phylip (3.68) and visualized by Treeview [Win32] version 1.6.6 (Figure 2).

Rigorous steps were taken to avoid risk of contamination of samples, or cross-contamination between samples. All the epidemiological samples were handled separately in a biosafety cabinet hood in a BSL3 facility to avoid any lab contamination. All RT-PCR and product analysis procedures described were independently repeated. Standard precautions to avoid product contamination were taken for all RT-PCR assays. Reaction mixtures for the reverse transcription and PCR stages were always prepared in a laminar flow-hood. The remainder of aliquots of the PCR master mixes were disposed after the first use. In addition to the use of non-infected control samples, all steps up to and including the analysis of the RT-PCR products were physically separated by working in a laboratory that did not use related products and all work was undertaken with designated equipment.

Analysis of Trend of JE in Uttar Pradesh

In order to investigate the possible trend of occurrence of JE, we compared the 2005 JE epidemic with the 1978 and 1980 epidemics, and also plotted a graph of cases/deaths and year of occurrence for 1992-2008. Data in this study was obtained from the World Health Organization (WHO) [2], National Institute of Communicable diseases (NICD) [8], National Vector Borne Disease Control Programme (NVBDCP) [9] and Indian Council of Medical Research (ICMR) [13]. Graphs were plotted between numbers of cases/deaths and time. WHO is the international surveillance agency and NICD, NVBDCP and ICMR are the Indian Government surveillance agencies that maintain such records.

Statistical analysis

The serological and CSF analysis were represented as arithmetic means (A.M.) ± SD. The data was analyzed using student t-test and χ^2-test $P < 0.05$. Rate of infection per million population (RM) and relative ratio (RR) was calculated as mentioned by Nash *et. al.* [14].

Results

Demographic characteristics

A demographic investigation of all 38

Table 2. Sequence information of JEV and other *flaviviruses* used in phylogenetic study.

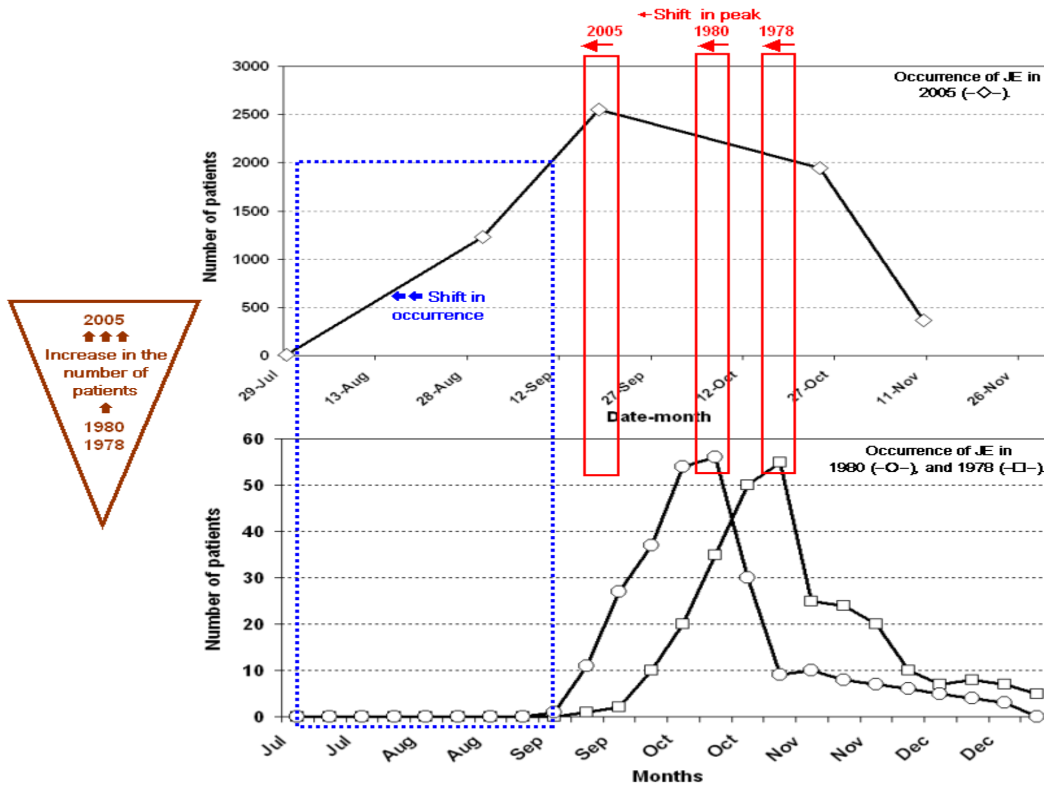
S.N.	Virus I.D.	Accession No.	Country	Year of Isolation	Host	Genotype
1	691004	Z34097	Sri Lanka	1969	Human	III
2	733913	Z34095	India	1973	Human	III
3	782219	U70402	India	1978b	Human	III
4	826309	U70403	India	1982a	Human	III
5	7812474	U70387	India	1978c	Human	III
6	B2524	U70392	Nepal	1985	Human	III
7	Beijing-1	L48961	China	1949	Human	III
8	Dengue	M29095	New Guinea	1944	Human	—
9	FU	AF217620	Australia	1995	Human	II
10	G8924	U70394	India	1956	Mosquito	III
11	GP-05	FJ979830	India	2005	Human	III
12	GP-14	DQ914531	India	2005	Human	III
13	GP-48	DQ914532	India	2005	Human	III
14	GP-55	DQ914529	India	2005	Human	III
15	GP-67	DQ914530	India	2005	Human	III
16	GP78	AF075723	India	1978	Human	III
17	GP-82	DQ914528	India	2005	Human	III
18	H49778	U70395	Sri Lanka	1987	Human	III
19	JaNAr0102	AY377577	Japan	2002	Mosquito	I
20	014178	EF623987	India	2001	Human	III
21	04940-4	EF623989	India	2002	Mosquito	III
22	057434	EF623988	India	2005	Human	III
23	JKT5441	U70406	Indonesia	1981	Mosquito	II
24	JKT7003	U70408	Indonesia	1981	Mosquito	IV
25	JKT9092	U70409	Indonesia	1981	Mosquito	IV
26	K91P55	U34928	Korea	1991	Mosquito	I
27	Kunjin	D00246	Australia	1960	Mosquito	—
28	MVE	AF161266	Australia	1951	Human	—
29	826309	U03689	India	1982b	Human	III
30	Nakayama	U03694	Japan	1935	Human	III
31	NO	L43566	Australia	1995	Human	II
32	P20778	Z34096	India	1958	Human	III
33	R53567	U70418	India	NA	NA	III
34	SLEV	NC_007580	USA	1975	Birds	—
35	Usutu	NC_006551	Australia	2001	Blackbird	—
36	WNV	AF206968	Egypt	1950	Human	—

Table 3. Demographic characteristics of patients (n = 38) with acute encephalitis in Uttar Pradesh (India), 2005.

S.N	Patient group	No. of patients	(%)	Population at risk (million)	Population at risk (%)	RM	RR	95% CI
A	JE (+ve) patients below six	7	18.4	14.015329	8.4	0.5	1.09	0.05-24.9
	JE (−ve) patients below six	5	13.2			0.36		
	JE (+ve) patient above 6	14	36.8	60.929021	36.7	0.23		
	JE (−ve) patient above 6	12	31.6			0.2		
B	JE (+ve) female patient below 6	1	2.6	6.771362	4.1	0.15	0.62	0.01-67.1
	JE (−ve) female patient below 6	2	5.3			0.3		
	JE (+ve) female patient above 6	7	18.4	29.059214	17.5	0.24		
	JE (−ve) female Patient above 6	6	13.2			0.21		
C	JE (+ve) male patient below 6	6	18.4	7.243967	4.4	0.83	1.24	0.78-1.97
	JE (−ve) male patient below 6	3	7.9			0.41		
	JE (+ve) male patient above 6	7	18.4	31.869807	19.2	0.22		
	JE (−ve) male patient above 6	6	15.8			0.19		
D	Total JE (+ve) female patient	8	21.1	35.830576	21.6	0.22	0.85	0.02-37.5
	Total JE (−ve) female patient	8	21.1			0.22		
	Total JE (+ve) male patient	13	34.2	39.113774	23.5	0.33		
	Total JE (−ve) male patient	9	23.7			0.23		
E	JE (+ve) male patient below six	6	15.8	6.771362	4.1	0.89	2	0.1-55.7
	JE (−ve) male patient below six	3	7.9			0.44		
	JE (+ve) female patient below six	1	2.6	7.243967	4.4	0.14		
	JE (−ve) female patient below six	2	5.3			0.28		
	Total population of U.P. *	166197921						

* Population figures are from the 2001 India Census.

Figure 3. Occurrence of Japanese encephalitis in Uttar Pradesh, India, in 2005 (-◇-), 1980 (-○-), and 1978 (-□-). (a) Presentation of cases (epidemic curve) of acute encephalitis in the 2005 epidemic. The graph represents the total number of cases (-◇-) reported during the acute encephalitis epidemic in 2005 in eastern Uttar Pradesh. (b) Presentation of seasonal incidence of acute encephalitis epidemics in 1980 (-○-), and 1978 (-□-). The plots (a, b) together suggest an increase in the number of patients (⬆), early occurrence (⬅) and early shift in peak (⬅) as in 2005 encephalitis started in late July and the peak (n = 2,554) timing of the epidemic was mid-September, while in 1980 encephalitis started in early September and the peak (n = 56) timing of the epidemic was early October, and in 1978 the epidemic started in late September and peaked (n = 55) in late October. (Values from ref. 2,7,13).



encephalitis patients was conducted and the population attack rate was calculated. Our study shows that the disease affected predominantly the low socio-economic, rural children aged 3 months to 15 years, in far-flung paddy fields, with a male to female ratio of 1.63:1. Estimated population at risk was 45.1% in the endemic areas. As per the 2001 census, the patients were classified into two groups (below and above six years of age) and RM and RR were calculated as presented in Table 3. RM suggests that patients below six years of age were more prone (RM 0.5) than patients above age six (RM 0.23). Males (RM 0.83) were found to be almost six times more susceptible to encephalitis than females (RM 0.15) below age six. Females above six years of age (RM 0.24) were more susceptible to JEV infection than females aged six or younger (RM 0.15). Data suggest that all patients and males below six years of age were more susceptible to JE infection, and males were more susceptible than females. The demographic features of encephalitis patients were consistent with JEV infection.

Clinical characteristics

This study suggests that acute encephalitis was marked by high-grade fever (100%), altered sensorium (100%), headache (71.4%), stiffness of the neck (47.6%), tremors (81%), vomiting (57.1%), altered mental status (47.6%), seizure (76%), myalgia (19%), abdominal pain (19%), depressed level of consciousness with coma, and paralysis followed by death. 76% patients suffered slurred speech (Table 4). Notably, the patients who survived showed evidence of mental retardation and/or neurological deficit. The clinical symptoms are suggestive of JEV infection among 21 patients out of 38 patients diagnosed as encephalitis.

Table 4. Presentation of clinical features in patients (n = 21) with Japanese encephalitis in Uttar Pradesh (India), 2005.

Clinical Characteristics	No. of Patients	(%)
Syndromes		
Encephalitis		
With weakness	7	33
Without weakness	6	28.6
Aseptic meningitis without encephalitis	6	28.6
Fever and headache	15	71.4
Signs and symptoms		
Fever (temperature > 37.8)	21	100
Weakness	15	71.4
Vomiting	12	57.1
Headache	15	71.4
Altered mental status	10	47.6
Stiff Neck	10	47.6
Myalgia	4	19
Tremor	17	81
Slurred speech	16	76
Abdominal pain	4	19
Seizures	16	76
Altered sensorium	21	100

The pathological investigations revealed that out of 38 cases, 55.3% (n=21) samples were positive for JE (Table 1) when tested by rapid IgM capture ELISA (JEV CheX) as described in materials and methods. Notably, during the epidemic of 2005, WHO reported only 37% cases of encephalitis were JE positive, which includes clinical as well as laboratory confirmed cases both [2]. A serological finding of our study is presented in Table 5. The infection could be characterized by marked pleocytosis in cerebrospinal fluid (90 ± 76.9 cells/cubic mm), and peripheral leucocytosis (64.7 ± 8.86% neutrophils) with mild anaemia (hemoglobin; 11.2 ± 3.25 g/dL). The data also exhibit alteration in lymphocyte (28.9 ± 8.4%) and polymorph (64.7 ± 8.86%). No significant difference was observed in white blood cell count (8416.7 ± 1192.6 cells/µL), eosinophil (1.95 ± 0.67%), reticulocyte count (1.92 ± 0.56%) and blood glucose (95.4 ± 25.1mg/dL). The CSF was collected within 2 to 7 days (4.2 ± 1.36 days) of onset of disease. Shifting of CSF protein content (49.5 ± 30.1mg/dL) towards the upper side of normal range (20.0-50.0 mg/dL) was also observed. Mild anaemia and leucocytosis with neutrophilia is one of the symptoms of JE infection. All the 21 cases, which were found JE positive by pathological investigations, were also confirmed to be JE positive by the serological investigation (Table 5).

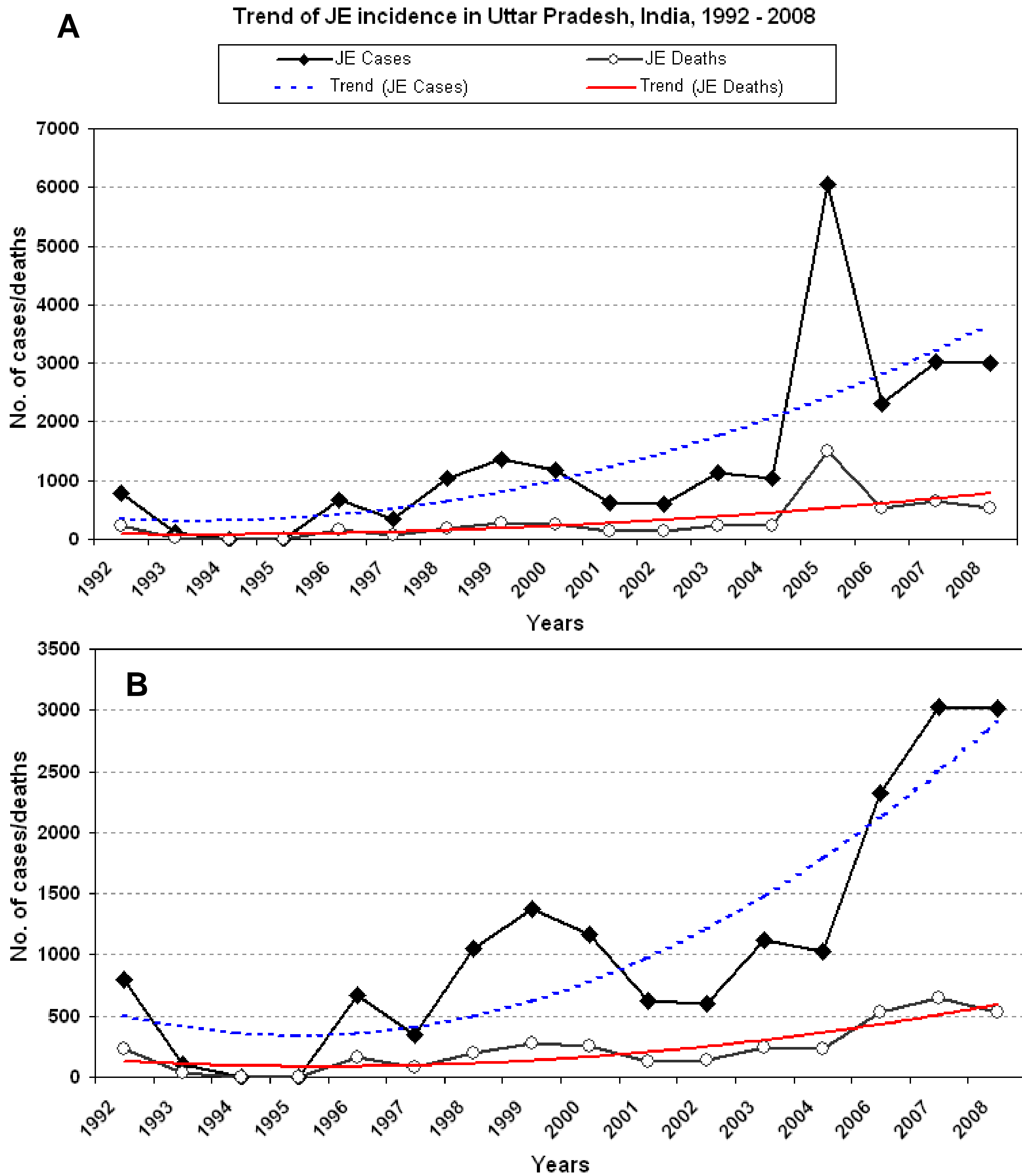
Phylogenetic analysis of GP05

The 2005 encephalitis epidemic surpassed all the pervious epidemics in its intensity; therefore, we sequenced GP05 and performed phylogenetic analysis. Our results (shown in Figure 2) suggest that the circulating strain GP05 was close to GP78 (the known endemic strain isolated in 1978) [7], and all the Indian JEV strains clustered together and fell in Genotype-III. The phylogenetic tree also proposes that the JEV strain 057434 isolated in 2005 from Gorakhpur (Uttar Pradesh), and 04940-4 isolated in 2002 from Bhandara (Maharashtra), are close to GP05. However, the JEV strain 014178 isolated in 2001 from Lakhimpur (Uttar Pradesh), is distinct from all the above-mentioned strains and lies between other 2005 strains and GP05.

Trend of JE occurrence

Since its first detection in India in 1955, JE has shown an increasing trend in its occurrence; however, it was not reported in Uttar Pradesh until 1978. There were two epidemics back to back in Uttar Pradesh in 1978 and 1980 preceded by heavy rainfall. We further compared the 2005 epidemic with the previous epidemics of 1978 and 1980 to predict the trend of JE epidemics in the area. In 2005, the encephalitis outbreak started in late July and peaked (n = 2,554) in mid-September, while in

Figure 4. Emerging trend of Japanese encephalitis in Uttar Pradesh between 1992-2008, including (A) and excluding (B) the 2005 epidemic. The graph represents yearly total number of JE cases reported (–♦–), total number of deaths (–o–), trend lines for total number of cases (-----) and deaths (—). (Values from ref. 2,8,9).



1980 encephalitis started in early September and then peaked (n = 56) in early October. In 1978 the epidemic started at late September and peaked (n = 55) in late October (Figure 3). These data indicate a significant increase in the number of patients, early occurrence (September to July), and early shift in peak timing (October to September). The duration of the infection has also increased from four months to five months (Figure 3).

During 1955 to 1965, a total of 52 cases were reported in India. During 1966 to 1975, reported cases numbered 763 with 325 deaths, whereas in 1976 to 1985 more than 8,000 cases with 2,600 deaths were reported in India [13]. To obtain more information about the infection pattern of JE, we

plotted a graph (Figure 4A) for the total number of JE cases/deaths in Uttar Pradesh for seventeen years (1992-2008) based on the numerical values available from NICD and NVBDCP. Our results (presented in Figure 4A) suggest that JE in North India is escalating and larger epidemics may occur in the future. We found a similar trend even when we excluded data from the 2005 epidemic (Figure 4B).

Discussion

Since the mid-1950s, JEV activity has been reported in various parts of India, both in the form of outbreaks of encephalitis and the level of antibodies found in the population [7, 15]. JEV, a flavivirus, is the major cause of encephalitis in Southeast Asia, including India, with high mortality. Among all viral encephalitis that is encountered in India, JE appears to be of greater significance. Only one of every 300 persons infected with JEV develops clinical encephalitis. The mortality in this disease has varied from 20-40% in different parts of India. The majority of the deaths occur during the first week of illness. A deadly outbreak of undiagnosed brain fever was reported in Uttar Pradesh and adjoining areas during 2005. In intensity and magnitude, it surpassed all the previously reported epidemics.

Our pathological investigations revealed marked pleocytosis in cerebrospinal fluid and peripheral leucocytosis with mild anaemia, which is in accordance with earlier studies done by Kumar *et al*. [16] in the same area. In CSF the elevated level of cells and alteration of protein level indicates penetration of the blood-brain barrier. The migration of neutrophils at the site of injury may be attributed to the production of soluble macrophage-derived factor (MDF), which is one of the key mediators in the host-innate immune response during JEV infection [17]. The mild anaemia may be because a result of MDF-induced hyperferretimia in serum [18]. The malfunctioning of the liver and kidney reported by Kumar *et al*. [16], may be caused by replication of the virus in these organs [19]. Presence of virus in CSF from most of the fatal cases might suggest that the virus is actively multiplying in the central nervous system. This also suggests that virus isolation or demonstration of CSF is an indicator of poor prognosis because some of the patients with low levels of IgM antibodies in CSF/serum expired. The present findings are comparable to the previous reports that isolation of virus from CSF and low levels of JEV-specific IgG and IgM in CSF and serum correlated significantly with mortality [16].

Table 5. Laboratory results of patients (n = 21) with Japanese encephalitis in Uttar Pradesh (India), 2005.

Variable	Mean ± SD	Range	Normal Range
Blood			
White -cell count (cells/μL)	8416.7 ± 1192.6	6300–11000	3530–13050
Hemoglobin (g/dL)	11.2 ± 3.25	6.0–17.5	11.5–18.0
Lymphocyte (%)	28.9 ± 8.4	13.0–43.0	25.0–50.0
Polymorph (%)	64.7 ± 8.86	50.0–83.0	30.0–60.0
Eosinophil (%)	1.95 ± 0.67	1.0–3.0	0.3–5.0
Reticulocyte Count (%)	1.92 ± 0.56	1.0–3.0	1.0–2.0
Blood glucose (mg/dL)	95.4 ± 25.1	60.0–140.0	70.0–140.0
Cerebrospinal fluid			
Interval between onset and collection (days)	4.2 ± 1.36	2–7	—
Cell count (per mm^3)	90 ± 76.9	10.0–260.0	< 4.0
Protein (mg/dL)	49.5 ± 30.1	20.0–100.0	20.0– 50.0
Glucose (mg/dL)	50.57 ± 9.4	36.0–68.0	40.0–70.0

Our demographic, clinical, pathological, and serological investigations confirm the circulation of JEV in Gorakhpur and adjoining areas. We have earlier reported the prevalence of JEV-GP78 in the affected area [7] and our present results (Figure 2) reconfirm the presence of GP78 in North India, especially in Uttar Pradesh during the JE outbreak of 2005. Clustering of JEV strains GP05 and 057434 (reported from another laboratory during the same epidemic) with GP78 again concurs with our results and also suggests that identification of GP05 by RT-

PCR is real positivity and not laboratory contamination during the PCR procedure. JEV strain 014178, isolated in 2001 from Lakhimpur (Uttar Pradesh), caused high fatality in pediatrics claiming 443 cases with 96 deaths (CFR 21.7%) [20]. Therefore, it appears that further evolution of the 2001 strain may have caused the large JE outbreak of 2005 with high fatality (CFR 22.9%). Similarly, JEV strains 057434 and 04940-4 caused high fatality and were also found to be closely associated with GP05. Few JEV strains isolated from the same epidemic differ from GP05 [21]. This may have two possible explanations: (i) the co-circulation of JEV GP05 with other already circulating strains in the area, and (ii) the lack of proofreading activity of viral RNA dependent RNA polymerase leading to a mutation in viral genome during RNA replication resulting in diverse evolution of 2001 JEV strain (014178). If this is the case, the possibility of further evolution/emergence of new JEV strains cannot be excluded. The presence of JEV strain 04940-4 in Bhandara, (Maharashtra State) in 2002 suggests that JE is spreading to new areas, which is an alarming concern.

Considering the large number of JE negative results, it is important to understand that different diseases of children affecting the brain and sensorium and causing death should not be clubbed together just because they occurred in the same time period, assuming that all of them represented one epidemic. There were a few cases with low platelet counts with fever. This clinical picture was clearly different from JE, and was most probably due to Dengue [3,16]. Earlier studies have shown that in addition to JEV few other serologically related flaviviruses, such as Dengue and West Nile viruses, are active in Uttar Pradesh [7]. Another reason for the large number of non-JE patients may be the low level of viremia and rapid development of neutralizing antibody. It is very difficult to detect genomic RNA or to isolate virus from serum samples; hence IgM capture ELISA for JE antibody or reverse transcription after virus culture are recommended [21,22,23]. However, cross-reactivity of antibody and mutation during culture passage and reverse transcription are the main drawbacks. These difficulties also emphasize the need for CSF collection for epidemiological studies and clinical diagnosis. Consequently, there is a need for effective and efficient methods for diagnosis and identification of JEV in patients.

Commencing in the last week of July, the 2005 encephalitis epidemic started about a month earlier as compared to the previous epidemics of 1978 and 1980 (Figure 3). The early detection of JE could be attributed to the improved surveillance/diagnoses, but ecological issues such as seasonal drift also need consideration. Even though the data points for the 1978 and 1980 epidemics are far less in comparison to the 2005 epidemic, the graph suggests that further investigation in the future will help us to understand JE circulation kinetics for better management during future epidemics. Interestingly, our graph (Figures 4A and 4B) suggests an escalating trend of JE year by year in Uttar Pradesh and indicates that there might be an even larger JE epidemic in the future. This observation is in accordance with a recent prediction of substantial risk of zoonotic and vector-borne emerging infectious diseases (such as JE) in North India, especially in Uttar Pradesh and adjoining areas [24]. Deeper insights are required to determine why JE is showing an intensifying trend in North India. The affected area in North India is known as the "Terai area." Floods are an annual feature in the region giving rise to water logging. The warm, humid climate of the region provides an excellent breeding ground for *Culex tritaeniorrhynchus* and *Culex vishnui* mosquitoes, which are vectors of JE. Therefore, during the rainy season, an increase in the population density of the mosquito in this division is observed. The area is densely populated so mosquito-human contact is very frequent. Villages in this rice-growing region abound in stray and reared pigs. Studies from peninsular and eastern parts of India indicate that pigs are the main vertebrate host of the virus and the major reservoir of the infection [25]. Pigs, besides other animals, are widely prevalent in both rural and urban areas of Uttar Pradesh. However, epidemiological and ecological aspects of the illness are yet to be studied in this part of the country. Due to the evolution of new viral strains and/or reemergence of older viral strains, children lack herd immunity. Although health management facilities are improved in the area, there is still a lack of adequate resources and proper facilities for health care and hygiene. These factors may be the responsible for the intensifying trend of JE in North India.

JE is still a major health problem in North India. No proper antiviral against JEV is available [26,27,28]. The following preventive measures may largely mitigate the disease: proper sample

collection; prediction of future strain(s); educating public health workers and professionals; vaccination; trials of new antiviral therapy; improvement in clinical management, especially early and specific detection; nutritional support to the affected patients. Wider issues, including current agricultural practices, water management systems, and human behavioral patterns, need to be investigated. Alterations in ecological issues such as seasonal drift, which may cause shifts in the early occurrence of the disease, should also be taken into consideration. There is a need to monitor JE in birds, mammals and its vector(s), along with human, to obtain the proper information about climatic, entomological, viral, and human host factors affecting JE. The remedy lies in tackling the cause and there is a need for a joint venture among health officials, researchers, clinicians and ecologists who can save lives by resolving the causative factors and solve the escalating problem of JE in India. To this end, a combination of strategies is required and we need to proceed with a sense of urgency in this matter.

Our results show that JEV infection led to marked pleocytosis in CSF and peripheral leucocytosis with mild anemia. All the JEV strains reported in India fall under Genotype III. The JEV strain circulating during 2005 was similar to GP78 and possibly originated due to the diverse evolution of the 2001 JEV strain. JE is also spreading to newer areas. The trend of JE suggested that the problem in North India is escalating and larger epidemics may occur in the future. Considering the possibility of a larger epidemic in the future, serious steps should be taken to combat JE, including the development of more efficient surveillance methods and differential diagnosis.

Gene Accession Number

The sequencing result leads to identification of 1524 bp ss-RNA (JEV GP05 envelope gene), which was submitted in NCBI with FJ979830 accession number.

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