Mass scale screening of common arboviral infections by an affordable, cost effective RT−PCR method

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ABSTRACT

Objective: To develop a rapid, cost effective RT−PCR method for the mass scale diagnosis of such diseases at the viremia stage to find out the actual disease burden in that area. **Methods:** For this purpose, cases with the history of only short febrile illness were considered. Thus 157 samples with the history of dengue/chikungunya like illness and only 58 samples with a history of acute encephalitis syndrome (AES) were selected. **Results:** Out of 157 samples, 42 and 74 were detected as dengue and chikungunya, respectively and out of 58 AES cases only 23 could be detected as Japanese encephalitis by this RT−PCR method. **Conclusions:** This cost effective RT−PCR method can detect the total positive cases that remain undetected by ELISA method. Moreover, this method is capable to detect the viral RNA from patients' sera even after the appearance of IgM antibody at one fifth costs as compared with the other commercially available kits.

1. Introduction

Arboviral (arthropod borne) infections are one of the most important public health problems all over the world including India. The members of this group have the ability to replicate both in arthropods and vertebrate. The former one has a greater chance to act as a vector for transmission of the viruses than the later one. In most instances, the virus is maintained in nature between the vector and animal host. Like in the case of Japanese encephalitis, pig–mosquito–pig and bird–mosquito–bird cycle is responsible for the maintenance of the virus in nature. Man is the 'dead end' host[1]. Sometimes, a human−vector−human cycle may occur where the reservoir may be in either humans or the vector, like in yellow fever, dengue and chikungunya cases.

The member of the genus *Flaviviruses* and *Alphaviruses* of the arboviruses accounts for important diseases in India[2]. The major arboviral infections that affect a significant portion of Indian populations every year are Japanese encephalitis, dengue and presently chikungunya with high mortality or morbidity. These are mosquito borne diseases. Density of the mosquitoes rises during monsoon and post monsoon period.

Japanese encephalitis virus is a member of the genus *Flavivirus* of the family, Flaviviridae, transmitted to humans by mosquitoes (*Culex* sp.) between wild and domestic birds and pigs[3].

Japanese encephalitis is numerically one of the most important causes of viral encephalitis worldwide, with an estimated 50 000 cases and 15 000 deaths annually. Most of China, Southeast Asia, and the Indian subcontinent are affected by the virus, which is spreading at an alarming rate. The mortality rate of this disease ranges from 0.3%−60%[4]. The morbidity especially in children and young adults (aged 2−15 years) is very high[5], although elderly people are not spared, if affected. It has been reported that those who recover have neurological sequelae in about 30% of cases[6]. Since the isolation of this virus in Japan in 1935, it has spread all over the world including India and has become a major public health problem[6]. In India, the existence of

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Japanese encephalitis virus was first reported serologically in 1954[7]. However, the disease was first recognized in India at Vellore in 1955[7]. Since then, outbreaks at different years have been reported in different states[8,9]. In West Bengal, the first major outbreak of Japanese encephalitis took place in the year 1973 in the district of Burdwan and Bankura where more than 700 cases and 300 deaths have been reported[10]. The recurrence of Japanese encephalitis epidemic in the district of Bankura and Burdwan in the year 1976[11,12] and again in the year 1987 and 1988, in the district of Burdwan[13], has been well documented.

Dengue virus, also a member of the genus *Flavivirus* with its four sero types is now classified within the Flaviviridea family[14] and transmitted mainly by *Aedes aegypti* mosquitoes. Among the four sero types, infection with any of them generally leads to a mild self limiting febrile illness *i.e.* dengue fever. Its typical symptoms include headache, a characteristic skin rash, joint pain and body ache. A more severe form of the disease involving vascular and haemostatic abnormalities leads to dengue hemorrhagic fever and dengue shock syndrome, which is responsible for a high mortality rate, especially in children[15]. Dengue virus is responsible for a growing health problem in the tropical and sub tropical countries. The global incidence of dengue fever and dengue hemorrhagic fever has increased dramatically in recent decades[15].

In India dengue was first isolated in 1946, and many epidemics have since been reported[16–18]. Dengue hemorrhagic fever was first reported in Calcutta, West Bengal, in 1963 again in 1964 and subsequently in Visakhapattanam in 1969 and in Jalore, Rajasthan in 1985. Delhi has experienced the major epidemic of dengue hemorrhagic fever in 1996[17,18]. In addition, outbreaks have also been reported at regular intervals from different states of India[19–21]. In West Bengal, recurrent outbreaks of dengue were reported in 2002, 2004 and 2007 from West Midnapore[22] and from Siliguri in 2005[23].

Chikungunya virus is one of the major viral pathogens throughout the world, causing severe morbidity in developed and developing countries. It is a mosquito borne virus, of the genus *Alphavirus*, which is transmitted to humans by *Aedes* mosquitoes[24]. Chikungunya virus causes an illness with symptoms almost similar to dengue fever except the prolonged arthralgia which persists for months to year[25], even after the disappearance of other clinical illnesses including the fever.

Chikungunya virus was first isolated from Tanzania in 1953[26]. In Asia, the first chikungunya outbreak was documented in Bangkok, Thailand in 1958, followed by other countries like Cambodia, Vietnam, Laos, Myanmar, Malaysia, Philippines, and Indonesia[27]. In India, the virus was first isolated in 1963–1965 in Calcutta[28]. The last outbreak of chikungunya virus infection occurred in India in 1971[29]. After that, no reports of such illness are available.

After a long gap, the present outbreak was first reported from the southern Indian state of Andhra Pradesh in November–December 2005[29]. An estimated 1.38 million people across southern and central India developed symptomatic disease during 2005 and 2006. By now it has affected a major part of the country very rapidly.

Japanese encephalitis, dengue and chikungunya are mosquito borne diseases. In case of Japanese encephalitis the disease is transmitted by the *Culex* mosquitoes, which breed in the stagnant water of the paddy field during monsoon and post monsoon period. But in the case of dengue and chikungunya, *Aedes* mosquitoes, mainly the *Aedes aegypti* plays the role of vector in India, which is the day biter, domestic and peridomestic in nature and breeds in the household containers. In all the cases, the vector density rises after the monsoon period. Not only that, people of the rural areas take up piggery and poultry as their economic support. These could be the amplifying hosts or reservoirs of some arboviral infections. For these reasons, the epidemics of those arboviral infections occur every year in India. To diagnose all these cases from the viremia stage, we aimed to develop and standardize a rapid, sensitive method at a low cost, for early confirmation of the cases to control the epidemic as well as to reduce the mortality rate, with a better patient care.

The reverse transcriptase polymerase chain reaction (RT–PCR) is one of the methods for the diagnosis of many diseases[30]. Many cases are remaining undiagnosed as most of them are subclinical with a history of mild and short febrile illness. All these cases can be detected by RT–PCR method to ascertain the total number of cases in the affected area. Here, we report a single step RT–PCR method for the mass scale diagnosis of common arboviral infections in West Bengal, India. This method is rapid and equally sensitive to detect the virus in the patient sera at an affordable cost, with a great public health importance.

2. Materials and methods

2.1. Clinical samples

A total of 472 acute blood samples were referred from different medical college hospitals and from district hospital in West Bengal from August 2010 to December 2010. Among them, 391 samples from the patients with high fever, rashes, joint pain/swelling and vomiting were selected for detection of dengue and chikungunya infections, if any. Rest of the 81 samples with the history of acute encephalitis syndrome were considered for Japanese encephalitis diagnosis. In all the cases, steady cold chain conditions were maintained during transport and repeated freeze–thawing was avoided for the protection of viral RNA.

2.2. Serology

For the detection of IgM antibody, ELISA test was performed, according to manufacturer's protocol. The kits were purchased from National Institute of Virology (NIV), Pune, India.

2.3. RNA extraction

The samples, having a history of short febrile illness (up to 48 h), were considered for the isolation of viral RNA. In this process, some IgM antibody positive samples were also included. RNA was extracted from the sera, using QIAamp® RNA viral kit (QIAGEN Inc., Valencia, CA), according to manufacturer's protocol.

2.4. RT–PCR

2.4.1. One step kit

To compare our single step method, two kits were used *i.e.* Qiagen one step RT–PCR kit and Access Quick RT–PCR system (Promega, USA), following the respective manufacture's protocol using RNA (50 pg to 1 μg) and 0.6 μM of the primer pairs. For Japanese encephalitis detection, newly constructed Japanese encephalitis virus specific forward primer 5′ CGA GAA CTT GGA ACA CTC ATT GA–3′ and reverse primer 5′– ATT GCC CAT GGT GAG CTT AGG ACA –3′ were used, whereas, chikungunya specific CNP1F 5′– GAA ATT GAT CCC GAC TCA ACC ATC C–3′ and CNP1R 5′–CCT TTA ATC GCC TGG TGG TAT AGC–3′[31] and dengue specific primers 5′–TCA ATA TGC TGA AAC GCG CGA GAA ACC G–3′ and 5′–TTG CAC CAA CAG TCA ATG TCT TCA GGT TC–3′[32] were used for its viral RNA detection.

2.4.2. Our one step protocol

One step RT–PCR method was standardized using 24 μL dH$_2$O, 2 μL of 10× PCR buffer, 0.65 mM dNTP (Invitrogen),

1.4 mM MgCl$_2$, 0.1 μL of 0.1 M DTT (Promega, USA), 0.125 μM primer pairs each, 0.5 U of AMV RT (Promega, USA), 0.5 U of Taq polymerase (Applied Biosystem) and 50 pg to 1 μg of RNA. The PCR cycle was set by initial heating at 42 ℃ for 1 h and 15 min at 94 ℃. The reaction was then run for 35 cycles at 94 ℃ for 30 sec, 54 ℃ for 1 min, and 72 ℃ for 1 min, followed by an additional elongation for 3 min at 72 ℃.

2.5. Cost calculation

The cost per reaction mixture was analyzed for each method. As the same amount of template was used in every method, so it was excluded in the calculation. In our one step method, 0.25 μM primer pairs were used in comparison to the kit based methods, where 0.6 μM primer pair was used (recommended). Considering the usage of the less quantity of the primers in our method, the unit cost was calculated excluding the cost of the primers.

3. Results

3.1. Serology

Out of 391 samples, 95 samples were positive to IgM antibody to dengue and 154 samples were reactive to chikungunya IgM antibody. Out of 81 acute encephalitis syndrome cases, 40 were Japanese encephalitis ELISA positive (Table 1).

Table 1
Result of IgM–positive and RT–PCR positive samples of confirmed Japanese encephalitis cases.

Method	No. tested	Positive to		No. tested	Positive to JE
		Dengue	Chikungunya		
ELISA	391	95	154	81	40
RT–PCR	157	42	74	58	23

JE: Japanese encephalitis.

Table 2
Comparative analysis of the unit costs of the newly developed procedure with the commercial kits.

Name of the kit		Cost of reagents (USD)	Total cost (USD)	Unit cost of reagent (USD)	Unit cost per method (USD)
Qiagen RT PCR kit		–	373.01	–	14.92
Access Quick RT–PCR kit		–	297.76	–	14.89
Our developed method		–	594.78	–	2.58
Materials used	10 mM dNTP (100 μL)	130.97	–	2.10	–
	DTT (500 μL)	62.69	–	0.01	–
	AMV RT (300 U)	229.84	–	0.38	–
	Taq pol (1 000 U)	171.28	–	0.09	–

3.2. PCR result

Out of 157 screened patient samples, only 42 and 74 samples produced dengue and chikungunya specific prominent band, respectively in 1.5% agarose gel, which amply proved those infections in them (Table 1 and Figure 1). Seven samples were also dengue IgM positive out of those 42 RT–PCR positive samples. A total of 23 samples produced Japanese encephalitis specific band by RT–PCR method out of 58 screened samples, of which four samples were Japanese encephalitis IgM reactive. In case of chikungunya, RNA could not be detected in IgM positive samples.
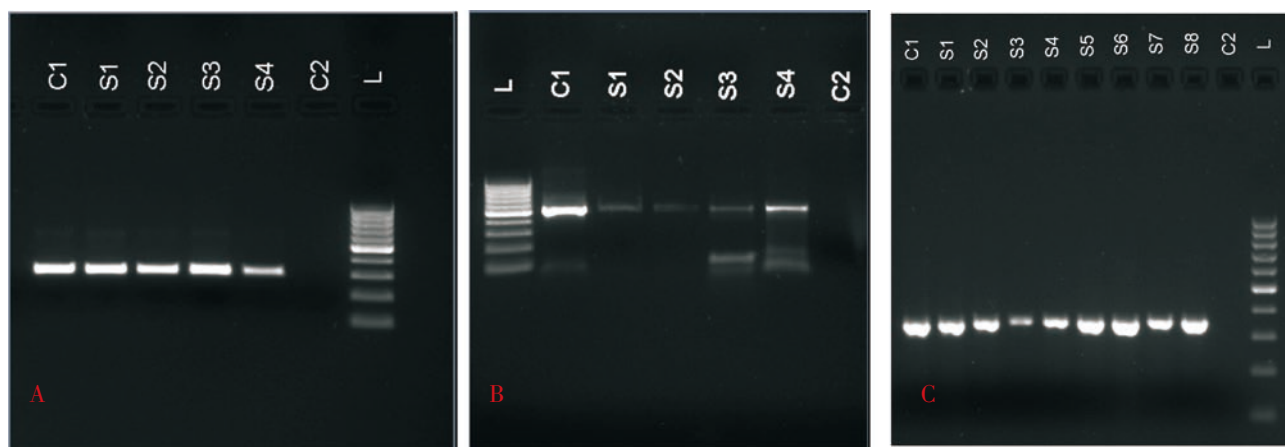
Figure 1. Results of agarose gel (1.5%) electrophoresis.
A: Showing band at 306 bp, obtained after amplification of CPrM gene of JEV from the patients′ samples; B: Showing dengue specific band at 511 bp;
C: Showing chikungunya specific band at 354 bp.
Lane L: 100 bp DNA ladder; Lane C1: Positive control; Lane S1–S8: Virus specific band at patients′ samples; Lane C2: Negative control.

3.3 Cost calculation

The calculated costs per reaction were summarized in Table 2. It is clear from the table that the cost per reaction of our developed one step method was the lowest as compared with the commercially available kits.

4. Discussion

The spread of these arboviral diseases throughout the country has become a major public health problem. As the vector density increases in the monsoon and post monsoon period, the transmission rate of the diseases rises sharply, which in turn increases the morbidity/mortality rate in West Bengal as well as in India. A huge number of febrile cases are referred to our laboratory. To assess all the cases, an economically feasible and potential process is required.

RT–PCR is one of the methods for the diagnosis of several viral diseases. It has also been successfully implemented for the detection of the members of the *Flavivirus* group[33]. WHO still considers RT–PCR as a standard method, for the detection arboviral infections. So early detection of Japanese, dengue and chikungunya cases by screening all the fever cases in the respected endemic areas, can control the spread of the diseases, which in turn will minimize the disease burden of that area. For this purpose, we conducted a validation study of PCR based detection of Japanese encephalitis, dengue and chikungunya viruses in clinical samples collected from different districts of West Bengal. This method is rapid, sensitive and cost effective, and can be employed for large scale detection of all the fever cases.

In our developed one step method, the cost of the test per sample claims almost one fifth in comparison with the widely available commercial kits like Qiagen one step RT–PCR kit and Access Quick kit. Although the initial investment of our developed one step method is higher, but for the mass scale screening of all the fever cases in the affected area, it ultimately lowers down the cost per reaction. Moreover, this method can detect the viral RNA at the late viremic stage, when the IgM antibody has already ushered in.

Hence, this cost effective, short term one step method constitutes a new report for the screening of endemic arboviral diseases by diagnosing all the positive cases and thus helps to measure the actual disease burdens which in turn have the great public health importance to control the epidemic of the respective diseases by adopting necessary measures.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Sabesan S, Konuganti HKR, Perumal V. Spatial delimitation, forecasting and control of Japanese encephalitis: India–a case study. *Open Parasitol J* 2008; **2**: 59–63.

[2] Raghunath D, Rao CD. Arthropod borne viral infections: current status and research. *Indian J Med Microbiol* 2008; **26**(4): 408.

[3] Hurk AF, Ritchie SA, Mackenzie JS. Ecology and geographical expansion of Japanese encephalitis virus. *Annu Rev Entomol* 2009; **54**: 17–35.

[4] Centers for Disease Control and Prevention. Question answer about Japanese encephalitis. [Online] Available from: http://www.cdc.gov/ncidod/dvbid/jencephalitis/qa.htm [Accessed on 12 December, 2010]

[5] Chatterjee S, Chattopadhyay D, Bhattacharya MK, Mukherjee B. Serosurveillance for Japanese encephalitis in children in several districts of West Bengal, India. *Acta Pediatr* 2004; **93**: 390–393.

[6] Dutta K, Rangarajan PN, Vrati S, Basu A. Japanese encephalitis: pathogenesis, prophylactics and therapeutics. *Curr Sci* 2010; **98**(3): 326–334.

[7] Reuben R, Gajanana A. Japanese encephalitis in India. *Indian J Pediatr* 1997; **64**: 243–251.

[8] Dhillon GP, Raina VK. Epidemiology of Japanese encephalitis in context with Indian scenario. *J Indian Med Assoc* 2008; **106**: 660–663.

[9] Kabilan L, Rajendran R, Arunachalam N, Ramesh S, Srinivasan S, Samuel PP, et al. Japanese encephalitis in India: an overview. *Indian J Pediatr* 2004; **71**: 609–615.

[10] Saxena V, Dhole TN. Preventive strategies for frequent outbreaks of Japanese encephalitis in Northern India. *J Biosci* 2008; **33**: 505–514.

[11] Banerjee K, Sengupta SN, Dandawate CN, Tongaonkar SS, Gupta NP. Virological and serological investigations of an epidemic of encephalitis which occurred at Bankura district, West Bengal. *Indian J Med Res* 1976; **64**: 121–130.

[12] Rajagopalan PK, Panicker KN. A note on the 1976 epidemic of Japanese encephalitis in Burdwan district, West Bengal. *Indian J Med Res* 1978; **68**: 3938.

[13] Mukhopadhyay BB, Mukherjee B, Bagchi SB, Chakraborty M, Mukherjee KK. An epidemiological investigation of Japanese encephalitis outbreak in Burdwan district of West Bengal during 1987–1988. *Indian J Public Health* 1990; **34**: 107.

[14] Curiel RF, Avilés HAE, Tovar R, Badillo AD, Nuez MC, Muñoz ML. The four serotypes of dengue recognize the same putative receptors in *Aedes aegypti* midgut and *Ae. albopictus* cells. *BMC Microbiol* 2006; **6**: 85.

[15] World Health Organization. *Dengue and dengue hemorrhagic fever.* Geneva: WHO; 2009. [Online] Available from: http://www.who.int/mediacentre/factsheets/fs117/en/ [Accessed on 20 March, 2011]

[16] Mohan RCV. Dengue fever in India. *Indian J Pediatr* 1987; **54**: 11–14.

[17] Singh NP, Jhamb R, Agarwal SK, Gaiha M, Dewan R, Daga MK, et al. The 2003 outbreak of dengue fever in Delhi, India. *Southeast Asian J Trop Med Public Health* 2005; **36**(5): 1174–1178.

[18] Gupta E, Dar L, Narang P. Sero diagnostic of dengue during an outbreak at a tertiary care hospital in Delhi. *Indian J Med Res* 2005; **121**: 36–38.

[19] Batra P, Saha A, Chaturvedi P, Vilhekar KY, Mendiratta DK. Outbreaks of dengue infections in rural Maharashtra. *Indian J Paediatr* 2007; **74**: 794–795.

[20] Thomas EA, John M, Bhatia A. Cutaneous manifestations of dengue viral infection in Punjab (north India). *Int J Dermatol* 2007; **46**(7): 715–719.

[21] Victor TJ, Malathi M, Asokan R, Padmanaban P. Laboratory–based dengue fever surveillance in Tamil Nadu, India. *Indian J Med Res* 2007; **126**: 112–115.

[22] Sarkar A, Taraphdar D, Chatterjee S. Investigations of recurrent outbreaks of unknown fever, estaeyblish rural dengue activity in West Midnapore, a costal district in West Bengal, India. *Arch Clin Microbiol* 2010; **1**(3).

[23] Taraphdar D, Sarkar A, Bhattacharya MK, Chatterjee S. Sero diagnosis of dengue activity in an unknown febrile outbreak at the Siliguri Town, District Darjeeling, West Bengal. *Asian Pac J Trop Med* 2010; **5**: 364–366.

[24] Pialoux G, Gaüzère BA, Jauréguiberry S, Strobel M. Chikungunya, an epidemic arbovirosis. *Lancet Infect Dis* 2007; **7**: 319–327.

[25] Powers AM, Logue CH. Changing patterns of chikungunya virus: re–emergence of a zoonotic arbovirus. *J Gen Virol* 2007; **88**: 2363–2377.

[26] Mavalankar D, Shastri P, Bandyopadhyay T, Parmar J, Karaikurichi V, Ramani KV. Increased mortality rate associated with chikungunya epidemic, Ahmedabad, India. *Emerg Infect Dis* 2008; **14**(3): 412–415.

[27] Chhabra M, Mittal V, Bhattacharya D, Rana U, Lal S. Chikungunya fever. *Indian J Med Microbiol* 2008; **26**(1): 5–12.

[28] Shah KV, Gibbs CJ, Banerjee G. Virological investigation of the epidemic of haemorrhagic fever in Calcutta: isolation of three strains of chikungunya virus. *Indian J Med Res* 1964; **52**: 676–683.

[29] Ravi V. Re–emergence of chikungunya virus in India. *Indian J Med Microbiol* 2006; **24**(2): 83–84.

[30] Ayers M, Adachi D, Johnson G, Andonova M, Drebot M, Tellier R. A single tube RT–PCR assay for the detection of mosquito–borne flaviviruses. *J Virol Methods* 2006; **135**: 235–239.

[31] Hasebe F, Parquet MC, Pandey BD, Mathenge EGM, Morita K, Balasubramaniam V, et al. Combined detection and genotyping of chikungunya virus by a specific reverse transcription–polymerase chain reaction. *J Med Virol* 2002; **67**: 370–374.

[32] Lanciotti RS, Calisher C, Gubler DJ, Chang GJ, Vorndam AS. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase–polymerase chain reaction. *J Clin Microbiol* 1992; **30**: 545–551.

[33] De Paula SO, de Melo Lima C, Torres MP, Pereira MR, Lopes da Fonseca BA. One step RT–PCR protocols improve the rate of dengue diagnosis compared to Two–Step RT–PCR approaches. *J Clin Virol* 2004; **30**: 297–301.