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RESEARCH ARTICLE

COMPARISON OF SHELL VIAL CULTURE ASSAY AND MOLECULAR METHODS INCLUDING A MOLECULAR CHIP FOR DIAGNOSIS OF ACUTE VIRAL ENCEPHALITIS IN NORTHERN INDIA

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ABSTRACT

Introduction: Viral encephalitis is potentially fatal disease which requires rapid diagnosis and management and should be ruled out from other infectious and noninfectious cases. Serological tests can only presumptively diagnose the condition. Cell culture methods are definitive diagnostic test but require comparatively longer time. Shell vial culture is better than conventional cell culture methods being more sensitive and rapid. Molecular methods like Polymerase Chain Reaction (PCR) can rapidly diagnose but its sensitivity needs to be evaluated. Finally newer microarray techniques can detect multiple viruses in a syndromic approach. So, this study was carried out to evaluate conventional cell culture, shell vial culture and PCR to diagnose viral encephalitis.

Materials and Methods: 203 consecutive clinically suspected patients with encephalitis, Dengue and Chikungunya fever were included. From each patient, blood and CSF samples were collected and inoculated on both conventional and shell vial culture. CSF samples were inoculated on RD (Rhabdomyosarcoma) for the Enterovirus isolation. PS (Stable Porcine cells) were used for JE. Serum samples were inoculated on C6/36 for Dengue virus and chikungunya virus isolation. All cell culture positive samples were subjected to molecular detection by RT-PCR. All the cell culture negative samples tested by a molecular chip developed by NIMHANS Bengaluru and XCyton Diagnostics Pvt Ltd Bengaluru.

Results: The positivity of conventional cell culture were 5 (2.4%), 6 (2.9%) 4(1.9%) samples respectively. In comparison, the positivity of shell vial cultures were 7 (3.4%), 10 (4.9%), 6 (2.9%) respectively. The corresponding positivity of RT-PCR were 3 (1.5 %) JE, 8 (3.9%) Enterovirueses , 4 (1.9%) Dengue and 0 chikungunya cases. All cell culture negative samples were negative by molecular chip methods except one sample which was positive for JC virus.

Conclusion: We recommend shell vial culture in diagnosis of viral encephalitis cases due to its better positivity and rapidity over conventional cellculture. It shows better positivity than RT-PCR.

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INTRODUCTION

Encephalitis is acute inflammation of the brain and encephalitis with meningitis is known as meningoencephalitis. Encephalitis can result from certain viral infections passed between humans, or from contact with infected animals or insects and they are known as viral encephalitis. Over 100 viruses are known to cause acute viral encephalitis in humans. Insect-transmitted viruses are the most common cause of epidemic viral encephalitis. Viruses that can be transmitted to humans by arthropod vectors are called arboviruses. They are the majority cause of viral encephalitis.

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In India, accumulation of water in a saucer-shaped landscape (*terai*) and extensive rice cultivation in eastern Uttar Pradesh and adjoining regions favour the growth of vector mosquito populations (Vaughn and Hoke, 1992). In addition, spread of waterborne pathogens is encouraged by poor hygiene and unsanitary conditions. The family Flaviviridae encompasses viral agents, which are important cause of arthropod-borne encephalitis in humans, specifically, Japanese Encephalitis (JE) virus which is the most common among them in the world (Vaughn and Hoke, 1992; Mackenzie *et al.,* 2004). It remains the most important cause of acute viral encephalitis and is a major cause of childhood mortality and morbidity in countries of Southeast Asia and Western Pacific regions (Mackenzie *et al.,* 2004). In India, JE is the leading cause of viral encephalitis, has been endemic in South India, since 1978 (Rao *et al.,* 2000). Other arboviruses like dengue and chikungunya

can produce acute encephalitis syndrome. Apart from JEV, Enteroviruses are known to cause severe neurologic diseases ranging from AFP (Acute flaccid paralysis) to encephalitis (Wildin and Chonmaitree, 1987; Kehle *et al.,* 2003). Enteroviruses belong to the genus *Enterovirus* in the family Picornaviridae, so called because "picorna" is the shortened acronym for the original members of the group (poliovirus, **c**oxsackie, **o**rphan virus, rhinovirus), and the fact they are all RNA viruses (Beaty *et al.,* 1955). Presently, the genus *Enterovirus* consists of more than 60 viruses that include poliovirus, coxsackie A and B, echovirus, enterovirus 70 and 71 (EV71). Enterovirus as a cause of neurological diseases is well known since the last century. Currently, in a developing country like India, serology is the mainstay of diagnosis of Dengue, Chikungunya and Japanese encephalitis. The rapid laboratory tools that is helpful in clinical situations of viral encephalitis include, demonstration of viral antigen, detection of specific IgM antibody and genome detection (Melnick *et al.,* 1979). Isolation in cell culture, though cumbersome, time consuming and less sensitive is still considered the gold standard for the diagnosis of flaviviral encephalitis (Melnick *et al.,* 1979). Shell vial culture (SVC) is a modification of the conventional cell culture technique for rapid detection of viruses in vitro. Rapid identification of cytomegalovirus in infected human urine specimens using Human Diploid Fibroblast (MRC-5) cells demonstrated its usefulness in diagnostic virology (Gleaves *et al.,* 1985). DEN-2, Japanese Encephalitis (JE) and West Nile virus are the Flaviviridae viruses for which the Shell Vial method has been tested (Jayakeerthi *et al.,* 2006). Diagnosis of viral infections of the CNS has been revolutionized by the advent of new molecular diagnostic technologies, such as the PCR to amplify viral nucleic acid from CSF (DeBiasi and Tyler, 1999; Thomson and Bertram, 2001; Weber *et al.,* 1996; Zunt and Marra, 1999). More recently, the emergence of PCR has revolutionized viral diagnostics by not only increasing detection sensitivity but also facilitating the detection of several viruses in parallel, either by multiplexing specific primers (for discrete viruses) or through careful design of degenerate primers (for members of a class). RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for viral genome detection and quantitation currently available. Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller samples. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell.

Despite the use of newer techniques, including CSF PCR, up to 70% of cases of encephalitis remain of unknown etiology in modern surveys (Glaser *et al.,* 2003). In more complex biological situations, such as diseases where many different viruses are present or where no etiologic agent has been identified, the limitations of even the best current methodologies become readily apparent. The existence of a large number of constantly evolving viral serotypes can render antibody-based detection nearly impossible. With PCR methods, because it is difficult to design compatible multiplex primer sets, the maximum number of viruses detectable in a single assay is relatively small (Broude *et al.,* 2001). Due to limitations of existing viral detection methodologies, the newly developed genomic approach to virus identification has been used in modern diagnostic laboratory. Using available sequence data from sequenced viral genomes, the newly developed technique, DNA microarray have the potential to simultaneously detect hundreds of viruses. The newest developments in DNA chip technology has several limitations including the complexity of fabricating the microarray, limited availability and high test cost but an excellent tool for rapid diagnosis especially for viral agents. So, this study was undertaken to evaluate various viral diagnostic techniques as for their rapidity, sensitivity, specificity and feasibility in viral encephalitis. A new molecular chip was also evaluated for rapid diagnosis of several viral etiology in encephalitis.

MATERIALS AND METHODS

This prospective study was conducted at Microbiology and Neurology department of from 1st November 2011 to 31st October 2013 in a tertiary healthcare center located in Lucknow, Uttar Pradesh, a town in Northern part of India. 203 consecutive clinically suspected patients with encephalitis, Dengue and Chikungunya fever were included. A pre-designed standard proforma was filled up for each patient.

Sample Collection: Appropriate samples were collected from each patient.

- **Whole blood:** Two sets of whole blood samples were collected in EDTA vials from each case. One set was used for viral culture while the other was utilized for molecular assays. Plasma and serum specimens were aliquoted in sterile vials and stored at -80ºC.
- **CSF:** It was obtained by lumbar puncture for Real time PCR (RT-PCR) .CSF was collected in a dry, sterile, screw cap container. It was aseptically divided into separate aliquots for examination for cells, Biochemistry, Microbiology and Virology. If any delay was anticipated, samples were held at 4°C for short term storage (1 to 3 days) or at or below –20°C for longer term storage.

Cell culture and Maintenance

Cell line: Stable porcine kidney cells (PS), Human Rhabdomyosarcoma cell line (RD) and C6/36 cell lines were obtained from National Center for Cell Sciences (NCCS), Pune, India. Cells were cultured in Eagle's minimum essential media (MEM) supplemented with 10% fetal calf serum (FCS) [Gibco BRL], penicillin/streptomycin (10000 U/ml); 100 mg/ml amphotericin B/ fungizone and L-glutamine (2 mM) at a constant temperature of 37^0C with 5% CO_2 [Sanyo, Japan].

Sample Preparation

All serum samples were diluted in 1:9 ratio in 2% MEM, filtered with 0.2 µM syringe filter (Whatman). Cerebrospinal fluid (CSF) specimens were inoculated directly into cells.

Sample Inoculation

For detection of Dengue and Chikungunya: C6/36 cells were seeded in shell vial at the conc. of 1.2×10^5/ ml, incubated in growth media (10% MEM) for 24 hour at 28 °C for monolayer prepration. (Fig.1)

- Removed the 2% MEM from shell Vial and add 200 µL of diluted and filterd serum samples.
- Centrifuged at 1600 rpm for 10 min.

- Tubes were incubated at 37 °C for 1 hour for virus adsorption.
- Added fresh 1 ml 2% MEM in each shell vial.
- The shell vial was incubated at 28 °C and observed it for CPE. (Fig. 2)

For detection of Japanese Encephalitis Virus and Enterovirus

- PS and RD cell line were used for detection of JEV and Enterovirus respectively were seeded at conc. of 0.9 × 10^6 / ml in 10 % MEM at 37 °C.
- After 24 hours removed the media and add 200 µL CSF.
- Centrifuged at 1000 rpm for 10 min
- Tubes were incubated at 37 °C for 1 hour for virus adsorption.
- Fresh 1 ml 2% MEM in each shell vial was added.
- The shell vial was incubated at 28 °C and observed it for CPE. (Fig. 3)

Molecular Methods for Viral detection

RNA extraction: RNA was extracted from the cell supernatant from the cell culture positive samples (Cells showing CPE) and directly from the CSF. RNA was also extracted from the serum samples that were cell culture negative. RNA has been extracted by QIAamp Viral RNA Mini Kit (QIAGEN) as per the manufacturer's protocol.

Real time RT-PCR for Chikungunya Virus

RT-PCR for Chikungunya virus was performed by the Geno-Sen's Chikungunya Virus Real Time PCR Kit for Rotor Gene 2000/3000/6000.

Real time RT-PCR for Dengue Virus

RT-PCR for dengue virus was performed by the Geno-Sen's DENGUE 1-4 Real Time PCR Kit for Rotor Gene 2000/3000/6000

Real time RT-PCR for Japanese encephalitis Virus

RT PCR was performed by the Geno-Sen's JEV Real Time PCR Kit for Rotor Gene 2000/3000/6000.

Real time RT-PCR for Enterovirus

RT PCR was performed by the Geno-Sen's JEV Real Time PCR Kit for Rotor Gene 2000/3000/6000.

Micro Array analysis

All the samples were evaluated on a Syndrome Evaluation System (SES) chip developed by NIMHANS Bengaluru and XCyton Diagnostics Pvt Ltd Bengaluru. In this novel technology simultaneous detection of 16 different viral pathogens (Japanese Encephalitis Virus, Dengue, West Nile, Enteroviruses as a group, Measles, Mumps, Nipah, Rubella, Rabies, Chandipura, Chikungunya, HSV 1 and 2, Cytomegalo Virus, Varicella Zoster, HHV-6, JC Virus) is possible in a single CSF sample.

This method has been standardized using the commercially available vaccines, panels and cell culture grown quantified viruses. The limit of detection (LOD) of the developed assay ranged between 0.1 viral particle to 50 viral particles per ml of CSF which is within clinically acceptable range along with 100% specificity.

RESULTS

Clinical features among suspected acute encephalitis patients

A total of 203 patients with acute encephalitis syndrome (AES) were admitted to the hospital from 1st November 2011 to 31st October 2013. Among the clinical features recorded, Fever (98.3%) and headache (83.9%) were most frequently observed while other features included vomiting (85.0%), altered sensorium (64.9%), convulsion (32.7%), meningeal signs (39.1%) and restlessness (21.3%) . Symptoms such as diarrhea, rhinitis, conjunctival congestion, hepatomegaly, splenomegaly and lymphadenophathy were rarely observed (Table 1).

Table 1. Clinical Features

Clinical Features	AES suspected viral etiology (n = 203)
Fever (%)	98.3 (200)
Headache (%)	83.9 (170)
Vomiting (%)	85.0 (173)
Altered sensorium (%)	64.9 (131)
Convulsion (%)	32.7 (66)
Meningeal signs (%)	39.1 (79)
Restlessness (%)	21.3(44)

Virus isolation by culture

Stable porcine kidney cells (PS), Human Rhabdomyosarcoma cell line (RD) and Vero cell lines was obtained from National Center for Cell Sciences (NCCS), India. All the cell lines were maintained and preserved in liquid Nitrogen. RD cells were used for the Enterovirus isolation. PS cells were used for JE and Vero cells were used for Dengue virus and chikungunya virus isolation. Before inoculation all the cells were checked for mycoplasma contamination. All the cell lines were negative for mycoplasma. All the CSF samples were cultured on RD and PS cells by conventional cell culture and shell vial culture assay. Only 5 CSF samples were showed cytopathic effect (CPE) on PS cells by conventional cell method while in shell vial 7 samples were positive. Six samples were positive on RD cells by conventional cell culture assay while 10 samples were positive by shell vial assay. Simultaneously all the serum samples were cultured on Vero cells by conventional and shell vial assay. Only 4 samples were showed CPE by conventional method while 6 samples were positive by shell vial culture assay (Table 2). The cell culture positive cases were further confirmed by reverse transcriptase polymerase chain reaction (RT- PCR).

Real Time PCR (rRT-PCR)

All the RD cell culture positive samples were subjected to enterovirus real-time PCR. Eight samples were positive for enterovirus. Samples positive in PS cells were subjected to JEV real-time PCR. Out of 7 samples only 3 samples were positive for JEV. Samples showing cytopathic effect in C6/36 cells were subjected to dengue and chikungunya virus real-

time PCR. Four samples were positive for dengue virus while all the samples were negative for chikungunya virus (Table 3).

Table 2. Virus isolation results of clinically suspected AES cases

Samples	Cells	No. of Samples	Conventional cell culture assay	Shell vial assay
CSF	PS	203	5(2.4)	7 (3.4)
	RD		6 (2.9)	10(4.9)
SERUM	C6/36	203	4 (1.9)	6 (2.9)

Table 3. Real Time PCR results of cell culture positive samples

	Virus	Real Time PCR positive
CSF	JE	3(1.5)
	EV	8(3.9)
SERUM	DEN	4 (1.9)
	CHICK	0

Molecular chip assay

All the cell culture negative CSF samples were subjected to microarray analysis by Syndrome Evaluation System (SES) chip. Results were negative for JE, EV, Dengue and Chikungunya viruses in all the samples. One sample was positive for JC virus.

DISCUSSION

Viral encephalitis is potentially life threatening disease which requires rapid diagnosis and management. Also It need to be ruled out from other infectious and noninfectious cases like cerebral malaria, stroke, diabetic coma etc. Conceptually, the most rapid diagnosis of an arbovirus infection can be made by direct detection of virus antigen or nucleic acid in clinical specimens (Shindarov *et al.,* 1979). Rapid serologic diagnostic tests, such as Enzyme Immuno-Assay (EIA) can provide strong presumptive etiologic evidence, if specific IgM is detected in the acute-phase serum or CSF specimen. Detection of IgM is not always evidence of current infection with certain arboviruses, especially flaviviruses, which can induce persistent IgM production (Lee *et al.,* 1992). Genome detection, though very useful in diagnosing WN encephalitis, is of limited value in diagnosing JE and Dengue encephalitis. Virus isolation, though more specific compared to antigen detection, is less sensitive and time consuming requiring minimum of 3–7 days compared to 5–6 hours required for antigen detection by indirect immuno-fluorescent technique. Shell vial culture, a modification of conventional cell culture technique works on the principle that centrifugation mechanical force enhances the viral infectivity to the susceptible cells. This technique has been used for the rapid diagnosis of infections by various viruses such as cytomegalovirus, Herpes Zoster, Mumps, Measles and respiratory syncytial virus (Gubler, 1998). In all these studies, shell vial culture technique has been shown to increase the rate of isolation of the viruses without any compromise on the specificity. Shell vial culture technique also has been shown to significantly reduce the time taken as compared to conventional cell culture technique. Shell Vial Culture could demonstrate viral etiology in all the 15 cases positive by Real time PCR for different viruses. In addition shell vial culture could detect the virus in 8 more cases, which Real time PCR failed to detect. Therefore, the technique could establish viral etiology in 23(11.3%) cases compared to 15(7.4%) by real-time PCR.

The significant reduction in the time taken (36 hours), compared to conventional cell culture technique (3–7 days) is an important advantage, which could be due to the hastening of viral entry into the cells of monolayer, as hypothesized. In order to ensure a rapid and precise diagnosis, early, prompt collection, transport and processing of the CSF samples becomes mandatory. Our study has demonstrated that the application of a rapid centrifugation assay for virus isolation improves the rate of isolation of these viruses. This assay represents an advance since it shortens the time needed to obtain results compared with the time required for the conventional tissue culture isolation method, it is easy to perform, and it is available in most diagnostic virology laboratories. Finally, this approach could be useful not only for increasing the virus isolation rate but also for obtaining a higher viral titer. We recommend the extension of the rapid centrifugation shell vial culture assay to the isolation of the other viruses. Cell culture negative samples were also negative by microarray analysis developed by NIMHANS Bangalore indicating good specificity of cell culture technique. One sample was negative by cell culture and positive for JC virus by microarray analysis. So, this Chip can be used to detect atypical viruses.

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