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Original Article

Microbiology Section

Diagnostic Approach to Viral Acute Encephalitis Syndrome (AES) in Paediatric Age Group: A Study from New Delhi

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ABSTRACT

Introduction: Acute Encephalitis Syndrome has heralded the emergence of multiple virulent pathogens, which may result in severe morbidity and mortality. In India, encephalitis is not notified and there has been a dearth of analysis for trends in encephalitis death rates and causation. A downward trend has been observed in encephalitis deaths, due to 'known' causes, which can be largely explained by improvement in diagnostic, treatment, and prevention methods. There is still a very high proportion of encephalitis deaths in developing countries, where the aetiological diagnosis of the pathogen is not established and thus, lies the importance of monitoring encephalitis morbidity and mortality with a view to improve pathogen diagnosis and identify emerging infectious diseases.

Aim: To formulate a diagnostic approach to viral acute encephalitis syndrome in paediatric age group.

Materials and Methods: A cross-sectional study including 50 paediatric patients, clinically diagnosed with acute encephalitis syndrome using WHO criteria was conducted. The CSF of all the patients was evaluated to diagnose the aetiology for viral pathogens. ELISA was used for diagnosing Japanese encephalitis and dengue encephalitis; and multiplex real time PCR was used for detecting HSV-1, HSV-2, Varicella zoster virus, Mumps virus, Enterovirus and Parechovirus.

Results: Confirmed diagnosis was established in 11 (22%) of 50 cases. A confirmed or probable viral agent of encephalitis was found in 7 (14%), bacterial agent was found in 2 (4%), non-infectious aetiology was found in 2 (4%). Fatal outcome was independently associated with patient age.

Conclusion: Despite extensive testing, the aetiologies of more than three fourth of the cases remains elusive. Nevertheless the result from the present study may be useful for future design of early diagnosis and treatment of the disease. New strategies for pathogen identification and continued analysis of clinical features and case histories should help us improve our ability to diagnose, treat and prevent encephalitis.

Keywords: Aseptic Meningitis, Encephalitis, Herpes Simplex virus

INTRODUCTION

Various clinical syndromes can be caused due to viral invasion of the Central Nervous System (CNS). These include syndromes such as encephalitis, meningitis, myelitis, and neuritis [1]. Encephalitis is defined as an inflammatory process of the brain parenchyma associated with clinical or laboratory evidence of neurologic syndrome [2]. Acute Encephalitis Syndrome (AES) is a group of clinical neurological manifestations. A wide range of viruses, bacteria, fungus, parasites, spirochetes, chemicals and toxins can be potential causes. It describes an acute, usually diffuse, inflammatory process that affects the brain. The clinical definition of a case of acute encephalitis syndrome is as follows: a person of any age, presenting at any time of the year, with an acute onset of fever and altered mental status manifesting with symptoms such as confusion, disorientation, coma, or inability to talk and/or new onset of seizures (excluding simple febrile seizures) [3]. Patients with suspected encephalitis often undergo prolonged hospitalization, may require undergoing a multitude of expensive diagnostic tests and frequently have poor prognosis with outcomes often including disability or death [4]. More than 100 different pathogens have been recognized as causative agents of AES of which the predominant causative agents are viruses. The most frequent pathogens are Japanese Encephalitis (JE), Herpes simplex, Varicella-zoster, Epstein-Barr virus, Mumps, Measles, Enteroviruses, Influenza, Adeno virus, Echo virus, *Mycoplasma pneumoniae*.

Aetiological diagnosis of acute viral encephalitis is made by qualitative and quantitative PCR and by antibody detection by ELISA. As acute encephalitis syndrome is caused by multiple organisms, it is very difficult and cumbersome to perform qualitative and quantitative PCR for individual viruses. The Real-time PCR assay has numerous advantages over PCR methods which are conventionally employed in labs. To list a few, it is superior in terms of rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity, and easy standardization. These advantages imply that nucleic acid-based assays or real-time quantitative assays might eventually replace virus isolation and conventional Reverse transcriptase-PCR as the new gold standard for the rapid diagnosis of viral infections, especially in the acute-phase samples [5]. Through this study, we proposed to standardize multiplex real time PCR for the detection of viral pathogens in Acute Encephalitis Syndrome.

OBJECTIVES

To determine the viral causative agents of the Acute Encephalitis Syndrome in the paediatric population using multiplex real time PCR (HSV1, HSV2, VZV, Enterovirus, Mumps, Parechovirus) and ELISA for IgM antibody against Japanese encephalitis virus and Dengue virus.

MATERIALS AND METHODS

A cross sectional study was performed at Department of Microbiology, Maulana Azad Medical College, New Delhi in association with Department of Paediatrics, Lok Nayak Hospital, New Delhi from January to December, 2015.

Inclusion Criteria

A total number of fifty paediatric cases (age> 28 days &< 12 years) who were clinically diagnosed with the Acute Encephalitis syndrome based on WHO definition for Acute Encephalitis Syndrome were

enrolled in the study. Assuming the prevalence of AES to be approximately 15% in paediatric population, with 10% allowable error, and 95% confidence interval, the sample size was calculated to be 49, according the formula, sample size (n) = (zα) 2 . p.q/l2. Hence sample size of 50 was taken, which included 50 suspected cases of acute encephalitis syndrome.

Exclusion Criteria

All the neonates and children above twelve years and clinically proven cases of pyogenic meningoencephalitis, tubercular meningoencephalitis, cerebral malaria, acute disseminated encephalomyelitis and encephalopathy due to some metabolic disease, Patients receiving any specific treatment like antibacterial, antifungal, anti-protozoal or antiviral drugs, sporadic brain attacks (cerebrovascular accidents, stroke), head injuries, intracranial space occupying lesions, Human Immunodeficiency Syndrome (HIV) and Acquired Immunodeficiency Syndrome (AIDS) were excluded from the study.

Sample collection and processing: One mL of Cerebrospinal Fluid (CSF) sample was aseptically collected from paediatric patients by lumbar puncture in a sterile leak proof container. Sample was transported to the laboratory immediately maintaining a cold chain using icepacks in a vaccine carrier. Sample was stored at -70°C if not processed immediately. Two mL of venous blood was collected aseptically in a plain vial. Serum was separated from the blood sample and aliquoted in Eppendorf vials and immediately transferred to -70°C until processed further.

All the CSF samples were subjected to multiplex real time PCR (Fast Track Diagnostics Viral meningitis Kit) as per manufacturer's instruction. They were also tested for Japanese encephalitis by ELISA against IgM antibodies (NIV Pune kit), Dengue Virus ELISA against IgM antibodies (NIV Pune kit) and Antimeasles Antibody ELISA as per manufacturer's instruction.

The CSF samples were also tested by putting conventional bacterial culture and fungal culture (Sabouraud's dextrose agar) to rule out any bacterial and fungal causes.

The CSF glucose is usually normal during viral infections, and reduction of CSF glucose levels relative to blood glucose is characteristic of meningitis due to bacteria, *Mycobacterium*, or fungi [6]. CSF was subjected to biochemical tests to observe for CSF glucose, CSF protein and CSF leukocytes. The clinical-epidemiological profiles of patients were observed.

STATISTICAL ANALYSIS

All the statistical analysis was performed by SPSS ver. 21. The quantitative variables were expressed as mean±standard deviations. A probability value (p-value) ≤ 0.05 was considered statistically significant.

RESULTS

Sociodemographic Characteristics of the Study Population

Out of the 50 subjects, 41(82%) were from Delhi, 3(6%) from Haryana, 2(4%) Punjab and 4(8%) were from Uttar Pradesh. Mean Age of study participants was 5.8±3.5 years (Mean±Standard Deviation). The average age of female subjects was 6.2±3.4 years and male subjects 5.4±3.7 years.

In the sample set 22(44%) were female subjects and 28(56%) were male subjects. In the cases where aetiology was not determined 18 out of 39 cases were female subjects (46.15%) and 21 were male subjects (53.85%). Cases where a diagnosis was established 4 out of 11 cases (36.36%) were females and 7(63.64%) were males. Overall in females, 4 out of 22 cases (18.18%) a diagnosis was established and in males this ratio was 7 out of 28 cases (25%).

Results of Testing

Six types of Tests were conducted on all the 50 samples. Multiplex real time PCR could establish a definite aetiological diagnosis in 4 (8%) of the cases. JE IgM Antibody Capture ELISA (MAC-ELISA), Dengue IgM ELISA and Anti Measles IgG ELISA all established a definite aetiological diagnosis in one case each (2% of sample set each). Bacterial Culture helped to determine specific aetiology in 2(4%) of the cases. Two cases were diagnosed as Metabolic Encephalopathy based on various Biochemical Tests. All the CSF fungal cultures came out to be negative. [Table/Fig-1] enlists the results obtained with each type of test conducted on the samples.

Test	Number of Samples tested	Diagnosis established	Undiagnosed
Multiplex real time PCR	50	4	46
JE IgM ELISA	50	1	49
Dengue IgM ELISA	50	1	49
Bacterial Culture	50	2	48
Fungal culture	50	0	50
Antimeasles IgG ELISA	50	1	49
Others (Biochemical Tests)	50	2	48
Overall Basis	50	11	39

[Table/Fig-1]: Diagnostic tests employed.

Based on the causal microorganism or underlying pathology the aetiology in the sample population was divided into the categories such as; viral, bacterial and metabolic aetiology. In 11 (22%) of the cases various aetiology such as viral, bacterial, metabolic encephalopathy was found. Whereas, in 39(78%) of the cases no aetiology was found [Table/Fig-2].

Type of Aetiology Determined	Number of Cases	Percentage of Sample
Viral aetiology found	7	14%
Bacterial aetiology found	2	4%
Fungal aetiology found	0	0%
Metabolic aetiology found	2	4%
No aetiology found	39	78%
Total sample size	50	100%

[Table/Fig-2]: Type of aetiology determined in samples.

The Specific causal organisms or Pathology as determined by testing were as follows:

HSV1 in 2(4%) of the cases;HSV2 in 2(4%) of the cases; Japanese encephalitis virus in 1 (2%) case; Subacute Sclerosing Pan Encephalitis (SSPE) in 1 (2%) case; Dengue Virus in 1 (2%) case; *Staphylococcus aureus* in 1 (2%) case; *Streptococcus pneumoniae*

Specific Aetiology	Number of Cases	Percentage of Sample
HSV1	2	4%
HSV2	2	4%
JE	1	2%
SSPE	1	2%
Dengue	1	2%
Staphylococcus aureus	1	2%
Streptococcus pneumoniae	1	2%
Metabolic Encephalopathy	2	4%
Not established	39	78%
Total Sample Size	50	100%

[Table/Fig-3]: Frequency of various Infectious/Non-infectious aetiology.
HSV1 = Herpes Simplex Virus 1; HSV2 = Herpes Simplex Virus 2; JE = Japanese Encephalitis; SSPE = Subacute Sclerosing Pan Encephalitis

in 1 (2%) case; Metabolic Encephalopathy in 2(4%) of the cases; and in 39 (78%) of the cases no etiology could be determined. The specific etiology as determined by the tests is enlisted in [Table/Fig-3].

Clinical symptoms such as fever, change in mental status, new-onset seizures and headache were observed in all 50 (100%) cases. Confusion and disorientation was observed in 40 (80%) cases whereas, 31 (62%) cases were unable to talk. Three cases (6%) presented with coma. Focal neurological signs were present in 18 (36%) of the cases and cerebellar signs were present in 6 (12%) of the cases.

CSF analysis may provide invaluable information about the nature of the infectious process and cytological changes in the CSF generally indicate the nature of the infecting agent. CSF cell pleocytosis is observed in encephalitis, lymphocytosis is seen in viral encephalitis. The CSF glucose is usually normal during viral infections, and reduction of CSF glucose levels relative to blood glucose is characteristic of meningitis due to bacteria, mycobacteria, or fungi [6].

In this study also, CSF findings were consistent with the diagnosis of acute viral encephalitis; with mean CSF glucose in the normal range i.e. 44.4±3.5 mg/dL.

The CSF Glucose/Blood Glucose Ratio, for various aetiologies, lay in the range of 0.56-0.64 for the sample set. The CSF Protein levels were also analysed and for the overall sample set the CSF protein levels were 87.9±21.9 mg/dL whereas, CSF Leukocyte levels were found to be 223.2±58 cells/mm^3. For the entire sample set 18 (36%) of the cases were non anaemic, 6 (12%) of the cases were mildly anaemic, 25 cases (50%) were moderately anaemic and one case of severe anaemia was observed.

DISCUSSION

Acute Encephalitis Syndrome due to various pathogens can present with very similar and overlapping clinical features and presentations. The viruses are important human pathogens, both in terms of their global prevalence as well as the life-threatening severity of the disease that they cause [7]. Whereas “classical” presentations of the infections caused by each of the viruses allow for fairly accurate clinical diagnosis, clinicians understand that there are significant overlaps in the signs and symptoms, which further highlights the usefulness of rapid, sensitive, and specific laboratory diagnosis. Clinical management of AES cases is mostly symptomatic; however, laboratory confirmation of the aetiological pathogen helps in prompt initiation of therapy especially in potentially fatal cases of bacterial meningitis. CSF chemical analysis may provide invaluable information about the nature of the infectious process and cytological changes in the CSF generally indicate the nature of the infecting agent. CSF cell pleocytosis is observed in encephalitis in general but, lymphocytosis is seen more frequently in cases with a viral aetiology. The CSF glucose is usually normal during viral infections, and reduction of CSF glucose levels relative to blood glucose is characteristic of meningitis due to bacteria, mycobacteria, or fungi [6].

The study populations of 50 paediatric patients of age group between one month to 12 years comprised of patients from Delhi, Uttar Pradesh, Haryana and Punjab. Delhi being a metropolitan city has tertiary care facilities that is why patients from these neighboring states attend the hospital as well. According to the aetiology determined after testing, nine of the eleven cases where a specific diagnosis was established were from Delhi. The Japanese Encephalitis diagnosis was made in a resident of Uttar Pradesh and one of the cases of metabolic encephalopathy was from Punjab. Sharma et al., reported that there is a paucity of data about the regional epidemiology and aetiology of AES in India [8].

In this study, CSF findings were consistent with the diagnosis of acute viral encephalitis; with mean CSF glucose lying in the normal range i.e. 44.4±3.5 mg/dL. The CSF protein findings were also towards the normal side or towards the high normal side with average protein content of fifty samples being 87.9± 21.9 mg/dL. The average CSF to blood glucose ratio was 0.60 which was consistent with the ratio found by Pleumpanupat et al., [9]. Alteration of CSF protein concentration in CNS infections is the most common and the least specific diagnostic marker, since it is observed in a wide variety of infectious and non- infectious conditions. CSF protein levels may be normal or slightly elevated in viral encephalitis, and moderately to significantly elevated in bacterial and tuberculous meningitis [9]. Albeit, identification of the aetiological agent is necessary to understand epidemiology, identify targets for immunization, chart preventive strategies, implement appropriate control measures especially in outbreak situations, and to help formulate rational empirical treatment especially for non-viral causes of AES. The aetiology of acute encephalitis syndrome was determined in 22% (n=11/50) of patients out of which only 8% (n=4/50) were determined using multiplex real time PCR. 14% of patients had a viral aetiology and rest 8% were diagnosed with some other aetiology. Of all viral aetiologies diagnosed, the cases diagnosed were that of HSV1 (2/11), HSV2 (2/11), Japanese encephalitis (1/11), dengue encephalitis (1/11) and SSPE (1/11). The other causes included two cases of bacterial encephalitis and two cases of metabolic encephalopathy.

Of all the cases diagnosed, multiplex real time PCR could determine the aetiology for four cases; and the other aetiologies were established with the help of other tests such as ELISA for IgM antibody against JE virus, ELISA for IgM against dengue virus, ELISA for IgG antibodies against measles virus, Bacterial Culture, and various biochemical tests. The multiplex real time PCR Fast track Diagnostics kit has an assay designed for simultaneous detection of HSV1, HSV2, Varicella Zoster Virus, Mumps virus, Enterovirus, Parechovirus. The assay targets the conserved regions of virus genomes to ensure consistent detection. As the study also involved a battery of tests like ELISA for dengue virus, Japanese encephalitis virus and processing the CSF sample for detection of bacterial pathogens, a diagnosis was made for more number of cases and the diagnostic capability increased 75% i.e., 3 more cases were diagnosed in addition to the four cases detected by multiplex real time PCR. This suggested that more number of cases can be diagnosed by using a more organized way of detection of pathogen, based on the clinical presentation of cases and using a battery of tests determined on the basis of the clinical presentation. Enteroviruses are the viruses most frequently isolated from the CSF while HSV isolation has been most successful from brain tissue [10]. In the current study, however, no enterovirus was found in any of the cases by multiplex real time PCR; which could be due to the reduced performance of the kit due to competition between primer and probe sets, or amplification of non-specific products. In another study conducted by Read et al., similar findings were reported for enteroviruses where they implemented the use of Light cycler for multiplex PCR for establishing the aetiology of viral infections in the central nervous system [11].

Upon gathering and analysing clinical history, one case of HSV2 positive child was detected who was just one-month-old; and had a history of his mother having genital lesions caused by Herpes during pregnancy. Also, another case of SSPE, that was found, had a history of measles attack at the age of 2 years and the patient was eight-year-old at the time of study. Thus, these findings emphasize the need for a detailed clinical history and examination of the patient.

Aetiological confirmation of the agent causing AES is one of the

major diagnostic challenges in clinical medicine and diagnostics [7]. Several factors contribute to the difficulty in rapid identification of the aetiological agent. Firstly, the number of infectious pathogens known to cause AES is sizeable; they include several viruses, bacteria, fungi, and protozoa. Secondly, several conventional methods available for testing are time consuming, expensive, lack adequate sensitivity and/or specificity; and may not be easily accessible. Thirdly, the small volume of CSF available from patients is quite often in sufficient for laboratory testing for all the pathogens. Lastly, at present, there is no single method available for simultaneous detection of all pathogens causing AES. The sensitivity of real time PCR for a specific clinical application depends on the setup of each diagnostic laboratory, in terms of volume of extract used in PCR. CSF samples typically have much lower viral loads. The success in identifying the aetiological agent in AES largely depends on the collection of appropriate specimens, prompt transport and storage, and the choice of diagnostic test used.

Isolation and identification of viruses from clinical samples is the most definitive mode of diagnosis [12]. PCR is an important laboratory tool for the diagnosis of the various CNS infections, especially, because existing diagnostic tools are comparably slower or provide only indirect evidence. Sufficient data, from various studies, has been accumulated to conclusively demonstrate that detection of several viral pathogens in the CSF by PCR based assays has now become a first line diagnostic tool, for most pathogens causing AES [13].

There are several advantages for a clinical virology laboratory of performing PCR with the multiplex system. The rapidity of establishing a laboratory diagnosis in case of a CNS infection is important because a conclusive laboratory finding establishing a specific viral aetiology in cases of suspected meningitis would greatly aid in determining the exact requirement of further investigations, reduce the health expenditures associated with conducting a multitude of tests, and alleviate the anxiety caused by an indeterminate diagnosis. When an unusual or unexpected result is obtained and a repeat test is required to confirm the diagnosis, a rapid assay has the advantage that it can be repeated during the same work day [12]. Hence it is essential to update the diagnostic protocol to a diagnostic tool which can detect multiple viral pathogens that can potentially cause acute encephalitis syndrome, in a single run. Hence, underlining the need to use multiplex real time PCR more commonly and improvising upon it to include more pathogens (without creating any significant competition between primer and probe sets or amplification of non-specific products).

Overall, the multiplex assay accommodates lower costs and faster turn-around times for the detection of common pathogens found in CSF. The sensitivity and specificity of multiplex assay surpasses that of culturing techniques [12]. It has shown comparable sensitivity to the singleplex Light cycler assays and improved ability to distinguish HSV-1 and HSV-2. In a study conducted by Wong et al., overall; the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for each target tested on the ABI 7500 are as follows: HSV-1 100%, 99.3%, 98%, 100% respectively; HSV-2 100%, 98.6%, 96.2%, 100% respectively; VZV 94%, 98%, 94%,98%, respectively, compared to culture [14].

The real time multiplex PCR has shown improved ability to distinguish between HSV-1 and HSV-2. This can be a useful tool in establishing the prevalence of important viral pathogens causing acute encephalitis syndrome.

LIMITATION

In present study, CSF was used for diagnosis. The sensitivity of isolation from CSF samples is low for most viral infections of the CNS, because small amounts of virus are shed into the CSF. Inadequate sample volume; and improper storage and transport of specimens also contribute substantially to poor virus isolation rates. Also, RNA viruses are highly susceptible to external environment making it difficult to detect RNA viruses in most samples. Usually enteroviruses are the viruses most frequently isolated from the CSF while HSV isolation has been most successful from brain tissue. In the current study however, no enterovirus was found in any of the cases by multiplex real time PCR; which could be due to reduced performance of the kit due to competition between primer and probe sets, or amplification of non-specific products.

CONCLUSION

Acute encephalitis syndrome contributes to significant mortality and morbidity in India. The aetiological agents are varied, and physicians treating such children often feel limited by the lack of availability of diagnostic testing for most of these agents. Therefore, it is essential to move the diagnostic interest to a diagnostic tool which can detect multiple viral pathogens which cause acute encephalitis syndrome in a single run. Thus, emphasizing on the need to use multiplex real time PCR and improvise upon it to include more pathogens without any significant competition between primer and probe sets or amplification of non-specific products. The current study of 50 cases that met our definition of acute encephalitis syndrome illustrates several notable features of the syndrome. First, there is a wide spectrum of causative agents, and the best diagnostic method varies from agent to agent. Second, an etiologic diagnosis remains inconclusive in many cases despite extensive testing, particularly when stringent criteria for linkage to CNS disease are applied. Finally, there are no clinical epidemiological features that clearly distinguish patients whose disease has infectious causes from those whose disease has non-infectious causes or from those who have an illness of unknown etiology. Overall, the multiplex assay accommodates lower costs and faster turn-around times for the detection of common pathogens found in CSF. The sensitivity and specificity of multiplex assay surpasses that of culturing techniques. It has shown comparable sensitivity to the singleplex Light cycler assays and improved ability to distinguish HSV-1 and HSV-2. Further investigations are required on better diagnostic applications of multiplex PCR for AES.

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