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A Critical Study on Duration of Excretion of Entroviruses in Children of Acute Encephalitic Syndrome: A Longitudinal Prospective Study

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Abstract: ***Introduction: Acute encephalitis syndrome (AES) is characterized by an acute onset of fever and clinical neurological manifestation that includes mental confusion, disorientation, delirium, or coma at any age excluding simple febrile convulsion. In India, agent causing encephalitis are mainly viruses including JE virus (JEV), Enteroviruses (EV-71, 76,89), Chandipura viruses (CHPV) responsible for major outbreaks in past many years. Other viruses are HSV-1, Measles, Mumps Chikungunya, Kyansanur Forest Diseases, West Nile virus, Nipah (NiV) and Rabies viruses have been reported sporadically from different regions of India. National Institute of Virology, Gorakhpur unit has reported an association between enteroviral infection and AES in the region suspecting that some of the AES cases are due to non-polio enteroviruses but the exact diagnostic marker of EV infection is still unknown. EVs are etiological agents of encephalitis outbreak in human beings. Enteroviruses are transmitted predominantly via the fecal-oral route.***

Keywords: AES, JEV, EV, CHPV and NiV

1. Introduction

Acute encephalitis syndrome (AES) is characterized by an acute onset of fever and clinical neurological manifestation that includes mental confusion, disorientation, delirium, or coma[1]. Viruses have been mainly attributed to be the cause of AES in India although other sources such as bacteria, fungi, parasites, spirochetes, chemicals and some toxins have been reported over the past few decades[1]. The causative agent of AES varies with season and geographical location, and predominantly affects population below 15 years[1]. The history of AES in India has paralleled with that of the Japanese encephalitis virus (JEV) since the first report in 1955 from Vellore, Tamil Nadu. The first outbreak of JEV was reported in Bankura district, West Bengal in 1973. Thereafter, sporadic cases of AES and outbreaks have been the leading cause of premature deaths due to the disease in India. Based on various surveillance reports and outbreak investigations, Joshi and co-workers[1] classified the history of AES in India into 3 phases: (a) period before 1975 when a few cases with JE etiology were identified; (b) between 1975 and 1999 when more JEV cases were reported with frequent outbreaks that resulted in the development of JE endemic regions near the Gangetic plains and in parts of Deccan and Tamil Nadu and (c) between 2000 and 2010 where a dramatic change was observed in the AES scenario with rise in non-JE outbreaks mostly caused by viruses such as Chandipuravirus (CHPV), Nipah virus (NiV) and other enteroviruses.AES was coined way back in 2008 by the World Health Organization to streamline the surveillance and research of AES in India. Over the years, the scenario of AES in India has improved significantly in terms of research and health care facilities.

National Institute of Virology (NIV)-Gorakhpur unit has reported an association between enteroviral infection and AES in the region suspecting that some of the AES cases are due to non-polio enteroviruses[6-7] but the exact diagnostic marker of EV infection is still unknown. EVs are etiological agents of encephalitis outbreak in human beings[9-12]. Enteroviruses are transmitted predominantly via the fecal-oral route. Gorakhpur regioncomprising seven districts of eastern U.P. viz. Gorakhpur, Maharjganj, Kushingar, Basti, Santkabir Nagar, Siddharthnagar, DeoriaandGopalganj along with West Champaran district of Bihar[2-5] is an endemic region for AES. This is an underdeveloped region, with one of the lowest per capita income (contributing to the overall status of people) in Uttar Pradesh, and is characterized by a unique ecosystem due to its location, rainfall patterns and the water logging pattern. Besides this, extensive cultivation of rice during monsoon facilitates suitable breeding conditions for mosquitoes and other vectors responsible for spread of viral infections.

2. Aims

Since route of the spread of enterovirus is mainlythrough feco-oral, a detailed study was required considering the duration of excretion of enteroviruses in stool specimens of the pediatric Acute Encephalitis patients of the study area following RT-PCR Technique along with statistical data analyses.

3. Review of Literature

Endemic regions of various viruses reported to cause AES in India. JEV has its endemic zones running along the Gangetic plane including states of UP (east), Bihar, West Bengal and Assam, and parts of Tamil Nadu. CHPV that hit the states of Maharashtra (in Nagpur district) and eastern part of Gujarat in 2003 has extended its influence in the states of Andhra Pradesh. NiV hit the south-east Asian countries, mainly Bangladesh. It had its first outbreak in Siliguri, West Bengal in 2001[13-15]. NiV again caused an outbreak in Nadia district of West Bengal in 2007. Enterovirus outbreak was first reported from Gorakhpur; UP in 2006and its adjacent

districts of Eastern Uttar Pradesh have been experiencing periodic outbreaks of encephalitis during monsoon since last three decades. Looking back at the available literature of the epidemic of encephalitis in the region of Eastern U.P., JE virus was implicated as the sole cause of encephalitis but now the whole group of enteroviruses is considered to cause the disease[16-19]. The reverse transcription-PCR test in cerebrospinal fluid showed positivity for EV in 66 (21.6%) of 306 patients in year 2006. Sequencing and phylogenetic analyses of PCR products form 59 (89.3% of 66 specimens showed similarity with EV-89 and EV-76 sequences[6]. During 2010, 7/302 (2.3%) CSF samples were shown to have presence of Enterovirus genome by RT PCR. Similarly, out of 642 Rectal swab tested 242 (37.4%) samples have shown presence of EV. Mainly EV 76/89. EV90, COX A11, B1, ECHO-19 were detected in these samples[8].

In a study by Beig FK et al[19]in western UP, the most common etiology of VE was found enterovirus 71 (42.1%), followed by measles (21.1%), varicella zoster virus (15.8%), herpes simplex virus (10.5%) and mumps (10.5%). Japanese encephalitis virus was not found in any case. Enterovirus 71 infection caused significant morbidity and mortality in children and generalized convulsions/altered sensorium along with muliti-organ involvement were the significant findings in patients with Enteroviral Encephalitis[20].The term enterovirus, as the name implies, contains species of the virus that are found throughout the gastrointestinal tract[21]. Enteroviruses of human origin are subdivided into five species based mainly upon sequence analyses. The former taxonomy for these viruses included the following: (1) polioviruses,types 1-3; (2) coxsackie viruses of group A, Types 1-24 (there is no type 23); (3) coxsackie viruses of group B, types 1-6; (4) echoviruses, types 1.33 (no types 10,22,23,or 28); and (5) enteroviruses, types[24-26]. All of these species have go the potential to cause neurologic disease[22-23]. In particular, there are some of the most common cause of both aseptic meningitis and encephalitis throughout the world.

Identification of etiologic agent in cerebrospinal fluid (CSF) is done by using reverse transcription- PCR (RT-PCR) and ELISA. Sequencing and phylogenetic analysis of PCR produces are used to compare the similarity between the other known strains and confirms its identity[25].

The highly conserved nucleotide sequences comprising 7 regions in the 5' NTR have been indentified which have been used for the development of primer and probes used in RT-PCR for the detection of the enteroviruses. The demographics of the various infection and diseases have some consistent characteristics. In particular, several factors, including age, sex, and socio economics status, have largely predictable effects. One of the important determinants of enteroviral (EV) infection outcome is age. Different age groups have different susceptibilities to infection, severity of illness, clinical manifestations, and prognosis following EV infection. The largest amount and duration of virus shedding occurs on primary infection with a given EV serotype. Because infection is so common, most primary infections occur during childhood. For these reasons, young children are probably the most important transmitters of EV, particularly within households[27].

Enteroviruses have a worldwide distribution. Within a given geographic locality, some serotypes may be endemic, with little or only gradual change in the range of serotypes present form year to year. In temperature climates there is increased circulation in summer and early fall. In contrast, other serotypes may be introduced periodically, causing epidemics, with very few isolations reported in intervening years[28].

In general, encephalitis and aseptic meningitis caused by EV appear to be most frequent among those 5 to 14 years of age rather than those older or younger. Enterorvirus infections are more prevalent among persons of lower socioeconomic status and those living in urban areas. In a study utilizing active surveillance of healthy children for EV infections in West Virginia during 1951 to 1953, the rate of isolations among children in a lower socioeconomic setting was two-to sevenfold higher than among children in a higher socioeconomic setting. A Similar study in Ghana during 1971 to 1973 further indicated that EV isolations were significantly more frequent among children in areas with poorer sanitation and in urban areas during both rainy and dry seasons[32-33].

4. Material and Methods

Study Population: Children aged 1-15 yrs. of age admitted inpediatric department (Nehru hospital´, B.R.D Medical College Gorakhpur U.P.

Duration of Study: From August 2011 to November 2012.

Study Design: Longitudinal prospective.

Inclusion Criteria
1) Children aged between 1-15 yrsfit in AES definition
2) Positive consent

Exclusion Criteria
1) Children aged <1yr. and >15yrs.with or without AES definition.
2) Children of seizure disorder and with febrile convulsion.
3) Negative consent

Sample Frame: AES numbers allotted in line listing of cases admitted at BRD Medical College were used for preparing of a sampling frame. Systemic randomization was done and every 6thpatient of sample frame was selected till a number of 200 study subject was reached.

Sample Size: sample size was calculated by using formula n=z2pq/d2
where, z = 1.96 at 95% confidence interval (prevalence of EV+vity in AES patients) p = 100-q, d = precision =10% (assuming excretion of EV by 50% of AES cases.), n = 3.86X50X50/100=96

Adding 100% for loss on follow-up noncompliance, the required sample size was estimated as 192(approx.200).

Study Tool
A case sheet was prepared in the department of pediatrics with inputs from NIV, Gorakhpur unit. The case sheet was

pretested on 10% of the cases, after making necessary modifications; the schedule was applied in the current study. The final schedule consisted of general information, history and general examination, diagnosis, investigations and follow-up.

Sample Collection

Cases with suspected viral encephalitis were studied for their clinical feature and recovery. The stool samples of admitted patients were collected. The rectal swabs were taken periodically at 1st (D1), 2nd (D7) and 3rd (D14) Day for RT-PCR for EV analyses.

5. Methodology

During the study, children were observed prospectively in the cohort entitled 'Duration of excretion of EV on stool in acute viral encephalitis patients (EV)'. The cohort were identified at the time of admission of the patients in Pediatric Department of B.R.D. Medical College, Gorakhpur . Based on sampling frame comprising of children aged 1-15 years fulfilling the inclusion criteria prepared from the AES line list was updated regularly. Two hundred cases (study subject) were enrolled from the sampling frame using systemic random sampling with sampling interval of 6 (Every 6thcase was enrolled for the study). Subsequently, 14 cases were excluded based on exclusion criteria and 10 were excluded due to denial of consent for the participation in the study by the parents of the patients. The rectal swab and stool samples of all subjects(n=176) were collected on the day of admission (D1), which were treated as 1stsample. Next sample were taken on Day-7 and Day-14 as 2nd and 3rd samples. 36 cases and 25 cases were lost during 1st and 2nd follow up respectively. A total (n=115) subject remain in the study. The duration of excretion of enterovirus in stool was studied by doing RT-PCR of rectal swabs. Significance of the above observations was analysed statistically.

Independent variables (age group/sex) and dependent variable (EV positivity in stool) was taken in to account to find out any association between age and sex with the duration of excretion of EV in the stool.

6. Limitations

1) The study was carried out between July to September, which coincided with the peak season of AES epidemic. No study is available to establish the cause of seasonal epidemicity of AES due to EV rather than due to predominance of a particular EV during epidemic seasons. This study has revealed only that unknown EV and may not be applicable to any other EV causing AES during non-epidemic season.
2) The RT-PCR technique used in the study had detected 5'NCR (Non-Coding Region) which is conserved to all groups of EV. It cannot differentiate between different groups of EVs (Cox.A virus and/or Echo virus).

7. Statistical Test of Significance

Logistic regression was applied to find odds ratio of EV positivity in relation to age group and sex. Chi-square test and McNemar test were performed to test significance of difference between proportions.SPSS-21 software was used for statistical calculation. P value less than 0.05was considered statistically significant.

8. Result

It was observed that at the time of admission 46.96 patients were excreting EV in their stool and onday7 this percentage declined to 25.22% while on day 14it was 18.26% (Table 1).However, there were 6 cases in which 1st samples were found to be negative but 2nd sample became positive. McNemar test analyses between first and second samples showed that a significant number of patients continued to excrete EV in their stool on day 7 of their admission. Likewise, in 4 cases 2ndsamples were found to be negative but 3rd samples became positive. Out of these 4 cases, in 2 cases 1stsample was positive, 2nd became negative and 3rd again became positive whereas in remaining 2 cases initial 2 samples were negative for EV and the 3rd one became positive. Statistical analysis between 2nd sample and 3rd sample revealed that the number of patients showing positivity for EV decreased from 25.22% to 18.26% while on applying McNemar test this declination was found significant ($p<0.05$).

Table 1: Rectal Swab Positivity

Result of RT-PCR	RECTAL SWAB (%) D1	RECTAL SWAB (%) D7	RECTAL SWAB (%) D14	P-value between sample D1 and D7	P-value between sample D7 and D14
+Ve for EV	54 (46.96)	29 (25.22)	21 (18.26)	0.001	0.001
-ve for EV	61 (53.04)	86 (74.78)	94 (81.74)		
Total	115 (100)	115 (100)	115 (100)		

The positivity for EV in 1ststool sample is maximum in 51.3% cases between 1-3 years of age group followed by 50.00%% cases in children of age group > 10 yrs. Likewise 48.61% males were excreted EV in their stool while 44.2% females were excreting EV. (Table-2).For statistical analysis, the cases were divided into two groups younger children (aged ≤ 6 years) and older children (aged > 6 years). To know whether age and sex play a role in determining the duration of excretion of EV in stool, Logistic Regression test was applied and it was found that(1)Odds Ratio for EV positivity in children of ≤ years and > 6 years was 1.06[95%CI 0.50-2.25] however the difference in odds of EV positivity in both the groups were non-significant (p=0.87); (2)Odds Ratio for EV positivity in

male and female=0.81 [95% CI 0.38-1.74] and the difference in odds of EV positivity in both the groups were non-significant [p=0.59];

Table 2: Distribution of Various Age Groups Excreting EV in their Stools

Age in yrs	Male AES cases				FemaleAES cases				Total AES cases			
	Total No. (%)	No.EV+ in 1st sample (%)	No.EV+ in 2nd sample (%)	No.EV+ in 3rd sample (%)	Total No. (%)	No.EV+ in 1st sample (%)	No. .EV+ in 2nd sample (%)	No. .EV+ in 3rd sample (%)	Total No .(%)	No. .EV+ In 1st sample	No. .EV+ in 2nd sample (%)	No. .EV+ in 3rd sample (%)
1-3	19 (100)	10 (52.6)	07 (36.8)	05 (26.3)	16 (100)	08 (50)	04 (25)	02 (12.5)	35 (100)	18 (51.3)	11 (31.4)	07 (20)
3-6	19 (100)	08 (42.1)	08 (42.1)	05 (26.3)	13 (100)	05 (38.46)	02 (15.3)	03 (23)	32 (100)	13 (40.6)	10 (31.2)	08 (25)
6-10	26 (100)	13 (50)	03 (11.5)	02 (07.2)	14 (100)	06 (42.9)	03 (21.3)	02 (14.3)	40 (100)	19 (47.5)	06 (15)	04 (10)
>10	08 (100)	04 (50)	02 (25)	02 (25)	00 (00)	00 (00)	00 (00)	00 (00)	08 (100)	04 (50)	02 (25)	02 (25)
Total	72 (100)	35 (48.6)	20 (19.44)	14 (19.44)	43 (100)	19 (44.2)	19 (44.2)	07 (16.28)	115 (100)	54 (46.9)	29 (25.2)	21 (18.26)

The positivity for EV in 2nd stool sample was maximum in 1-3 years of age group (31.43%) followed by 31.25% in children of 3-6 year of age . It was observed that 19.44% males excreted EV in their stool as against 20.93% in females(Table-2). Logistic Regression of EV excretion in 2nd stool samples (dependent variable over age group and sex(independent variables), the test revealed that (1)Odds Ratio of EV positivity in younger children and older children=0.41[95% CI 0.16-1.04]; p=0.06 [non-significant]; (2)Odds Ratio of EV positivity in male and female = 0.58 [95% CI 0.23-1.46]; p=0.246 [non-significant].

The positivity for EV in 3rd stool sample is maximum in 3-6 year and in >10 years of age group (25.00% in each) followed by 1-3 year of age (20.00%).When duration in context with sex was recorded, it was found that 19.44% males were excreted EV in their 3rd stool samples in comparison to16.28%in females (Table-2).

Logistic Regression of "EV excretion in 3rd stool samples" (dependent variable over age group and sex (independent variables) revealed that odds of occurrence of EV positivity in 2nd week at younger children was lower than the odd in older children 0.478 [95% CI 0.254-1.936]. However again the difference was non-significant (p=0.494).Likewise, Odds of occurrence of EV positivity in 2nd week in male children was lower than the Odd in female children 0.701 [95% CI 0.254-1.936]. However again the difference was non-significant (p=0.494).

The study showed that age and sex do not play a role in determining duration of excretion of EV in stool. It is summarized as (1) Odds Ratio of EV Positivity in younger Children and older Children was = 0.478 [95% CI 0.168-1.357]; p=0.166 [non-significant] and Odds Ratio of EV positivity in male and female was = 0.701 [95% CI 0.254-1.936]; p=0.494[non-significant].

9. Discussion

An attempt has been made to assess the durations of excretion of enterovirus in stool of pediatric acute viral encephalitis patients. National Institute of Virology, isolated 66 cases of Enterovirus-76 in the year 2006. Therefore, greater attention has been paid towards enteroviruses as a suspected emerging cause for AES.

Enteroviruses excreted in human feces and urine, may present in treated waste water which can contaminate rivers, recreational waters and seawater. These enteroviruses may subsequently infect humans form water though many potential routes[29]. Hence, prolonged excretion of EV may act as an on-going source of infection.

Chung et al[30] found that the enterovirus could not be identified form the throat samples after 2 weeks of infection; however, its excretion through stool can persist up to 11 weeks. Furthermore, most enteroviruses can be isolated form stool samples even at 2-3 months after a nonspecific illness. In the present study, fecal excretion of EV during the course of AES has been examined using serial rectal swab collections. Although there have been reports on detection of EV in stool using polymerase chain reaction (PCR), the duration of fecal EV shedding in AES has not been well described. The reverse-transcription (RT)-PCR for the detection of fecal EV RNA in serial rectal swab samples were performed in this study.

It is a preliminary study, where patients with AES have been studied for duration of excretion of enterovirus in their stool (Rectal Swab) and also analyzed whether this duration has any correlation with severity of the disease. More and More studies are required from different parts of India to analyze the exact scenario of the disease.

The EV positivity in periodic sample of stool revealed a statistically significant decline in the percentage of cases excreting EV in their stool with the passage of time [The positivity in 1st sample was 46.96% which decline to 25.22%in 2nd sample and 18.26% in 3rd sample]. This shows that as the time passes, the chances of getting virus in stool becomes less.Wei Xu et al[31], Jun Han[34]and co-workers have also reported the same results in EV71 excretion in stool in patients suffering from Hand Foot Mouth disease. During the present study in D7 samples, 6 cases became positive which were negative at D1, i.e. at the time of admission. Likewise D7 data recorded 2 such cases in which D7 samples were negative while D1 and D14 samples were

positive. Similarly in 2 cases D1 samples were negative for EV which became positive at D14. Consequently, children excreting EV intermittently for a long time may serve as reservations of the virus and thus may contribute to the AES epidemics.

The current study did not show any relationship between duration of EV excretion in the stool with age and sex. The odds ratio of age group ≤6 years and >6 years with EV positivity in stool was 1.06[95%CI 0.50-2.25] in 1st sample, 0.41[95%CI0.16-1.04] in 2nd sample, and odds ratio for sex with EV positivity in stool was 0.81[95%CI 0.381-1.74 in 1st sample, 0.58[95%CI 0.23-1.46] in 2nd sample, and 0.701 [95%CI 0.254-1.936] in 3rd sample respectively.

10. Conclusion

At the time of admission 46.96% patients were excreting. EV in their stool, after 7 days of admission it was 25.22% and after 14 days 18.26% patients were excreting EV in their stool. With the passage of time the chance of getting EV in the stool becomes low and the excretion was not continuous but intermittent in nature.

11. Competing Interest

None

12. Funding

None

13. Ethical Issues

Ethical clearance obtained from ethical committee of BRD Medical College, Gorakhpur.

Disclaimer: The views expressed in this paper are those of the authors and do not necessarily reflect the official position of the institution they are affiliated with.

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