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Original Research Article

Prevalence of Enteric Adenovirus among Non-Rotavirus Diarrhea in Assam, Northeast India

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ABSTRACT

Introduction: Globally enteric adenovirus has become the second leading cause of viral diarrhea. Present investigation was undertaken to understand the adenoviral etiology of diarrhea among hospitalized children in Assam, northeast India.

Materials and methods: Over a period of two years (2013-2015), 455 faecal samples were collected from hospitalized children aged <5 years with diarrhea from Assam, northeast India for screening of rotavirus antigen. A total of 238 samples were rotavirus antigen negative and are screened for adenovirus antigen by ELISA and followed by PCR.

Results: Present investigation revealed enteric adenovirus infection in hospitalized children <5 years to be 10.9% (26/238). Prevalence was higher among infants (16.6%) and females (15.1%). Moreover, severe diarrhea (≥10 times) 14.9% (*P*=0.047) and restlessness 50% (*P*=0.002) is significantly higher in enteric adenovirus infected children. Enteric adenovirus infection was observed sporadically throughout the year; however infection was higher during warmer months (17%) than cooler months (7.3%) (*P*=0.0001). Adenovirus sequence analysis of the hexon gene showed adenovirus F type-41 to be the predominant serotype circulating in the study region. Analysis of the adenovirus fiber protein of the present study confirmed 15 amino acid deletions from the 15th repeat motif of the shaft region.

Conclusions: Present data demonstrated that enteric adenovirus could be considered an important viral etiological agent for acute gastroenteritis among children in northeast India along with other etiology.

Key Words: Acute Gastroenteritis, Adenovirus, Diarrhea, Northeast India, Prevalence.

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INTRODUCTION

Diarrhea is the second leading cause of death among children below five years and accounts for approximately 2.6 million deaths worldwide.[1] According to WHO, over 70% of diarrheal related deaths among children occur in South Africa and South East Asia.[1] Viruses are considered to be the major cause of acute diarrhea in young children.[2] Four categories of viruses are considered to be important agents of viral diarrhea: group A rotavirus (RVA), norovirus (NoV), adenovirus (AdV) and astrovirus (AstV).[3] Among viral diarrhea, rotaviruse is considered to be the major etiological agent in causing children diarrhea.[4] Human adenovirus (HAdV) detected worldwide in sporadic as well as in outbreaks cases of gastroenteritis and is considered to be second common agent of acute gastroenteritis (AGE).[5-8]

Adenovirus belongs to family *Adenoviridae* and genus Mastadenovirus.[9] To date, there are 60 serotypes of adenovirus identified and grouped into seven species A to G on the basis of haemagglutination, serum neutralization and genome analysis.[10,11] The disease pattern of adenovirus varies species to species. Adenovirus species F mainly type 40 and 41 are found to be regularly associated with gastroenteritis and are grouped in enteric adenovirus.[12] It accounts for 20% of the diarrheal diseases globally especially in children below five years. Other species such as A, C, and D have also been associated with diarrhea.[13]

Accurate understanding of the relative prevalence of enteric adenovirus in hospitalized diarrheal cases is important for effective management and preventive measures. In North East India, diarrheal diseases caused by viral etiologies have not been studied thoroughly and currently no data are available regarding the specific contribution of enteric adenovirus among children diarrhea. To address these knowledge gaps, the present study

was undertaken to determine the relative prevalence and characterization of enteric adenovirus infection among hospitalized children aged 0-59 months with diarrhea in Dibrugarh, Assam, Northeast India. Furthermore, the seasonality of enteric adenovirus infection from this region of India was also investigated.

MATERIALS AND METHODS

Sample Collection

Samples were collected from a tertiary hospital of Northeast India, Assam Medical College and Hospital, (AMCH) Dibrugarh, Assam for screening of rotavirus antigen (Ag) under the study National hospital based rotavirus surveillance network. A total of 455 fecal specimens were collected from children aged below 5 years who were hospitalized for diarrhea during the period from May 2013 to April 2015. A total of 238 cases negative for rotavirus diarrhea during the period were screened for enteric adenovirus Ag. The inclusion criteria of the cases were hospitalized children aged < 5 years with acute watery diarrhea, with or without vomiting and abdominal pain. Written informed consent was taken from either the guardian/parents of the patient participating in the study in a protocol approved by the Institutional Ethical Committee of Regional Medical Research Centre (RMRC) for Northeast Region, Indian council of medical research (ICMR).

Enteric Adenovirus Ag Detection

Preliminary screening of the rotavirus negative fecal samples for the detection of enteric adenovirus Ag was performed using Adenovirus Ag ELISA kit (DRG Instruments GmbH, Germany) in accordance with the manufacturer's instructions.

Viral DNA Extraction

For viral DNA extraction, 30% (v/v) suspensions of adenovirus Ag positive stool samples were prepared in sterile Dulbecco's Modified Eagle Medium (DMEM) - low glucose (Sigma, MO, USA) by addition of 1.5 g of semi-solid faces or approximately 1.5ml of liquid feces in 3.5ml of DMEM. The suspension was vortex thoroughly for 1 min and centrifuged at 10,000 rpm for 10 min at 4°C. The clarified supernatant was collected carefully and stored in aliquots at -20°C until further processing. For molecular typing, every enteric adenovirus Ag positive supernatant was used for viral DNA extraction by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions.

Enteric Adenovirus Detection by PCR for Hexon and Fiber Genes

PCR was carried out in 50 µl reaction mix containing5 µl of viral DNA template, 25 µl of 2X PCR master mix (Promega, USA), 1 µl each of adenovirus hexon gene primers (20 pmol/ml) Ad1-5'-TTCCCCATGGCICAYAACAC-5', Ad2-5'-CCCTGGTAKCCRATRTTGTA-5'and fiber gene F specific primers (20 pmol/ml) AdF1-5'-ACTTAATGCTGACACGGGCAC-3' and AdF2-5'-TAATGTTTGTGTTACTCCGCTC-3'.[14] The volume was made up to 50 µl with distilled water. Thermal profile consisted of an initial denaturation step at 94 ℃ for 5 min followed by 30 cycles at 94 ℃ for 1 min, 54 ℃ for 45 sec, 72 ℃ for 2 min with final extension at 72 ℃ for 7 min for both hexon and fiber gene regions. PCR products were loaded on to 1.5% agarose gel containing ethidium bromide (0.01 mg/ml) along with 100 bp molecular size ladder and viewed in a gel-documentation system.

The PCR products were purified using High pure PCR product purification kit (Roche, Mannheim, Germany). Purified PCR products were sequenced using dideoxy cycle sequencing (Sanger) reaction in both directions in a 3130 Genetic Analyzer (PE Applied Biosystems, Hitachi, Japan).

Nucleotide Sequence Accession Numbers

Accession numbers of the adenovirus hexon gene and fiber gene sequences submitted to GeneBank are: KU904307 to KU904313 and KU681045 to KU681056 respectively.

Phylogenetic Analysis

Nucleotide sequence BLAST search was performed using the National Centre for biotechnology Information (NCBI, National Institutes of Health, Bethesda, MD) Basic Local Alignment Search Tool (BLAST) server on GenBank database release 143.0.[15]

Adenovirus hexon gene and shaft region of fiber gene sequences were aligned separately using Clustal W (codons) application in Molecular Evolutionary Genetics Analysis (MEGA) version 7.0.[16] Moreover, model selection of the aligned sequences was also performed in MEGA 7.0. As per the best-fit model the evolutionary history was inferred using Maximum Likelihood method based on Tamura 3-parameter model for hexon gene and Hasegawa-Kishino-Yano modelfor fiber shaft region.[17,18]

Adeno virus hexon gene and shaft region of fiber gene analysis involved 20 and 23 nucleotide sequences of which 7 and 12 sequences are of the present study respectively, while rest are most nearest respective related sequences and reference/prototype sequences from GenBank.

Statistical Analysis

Standardized questionnaires for diarrheic cases, enteric Adenovirus Ag ELISA and sequencing result reports were used to generate data for statistical analysis. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 20.0 (trial version). Differences in proportions of enteric adenovirus positivity in different age, sex, season and genotype were tested using the Pearson Chi-square (χ^2) test. Odds ratio and their 95% confidence interval (CI) were calculated to measure the magnitude and direction of association. A cut off *P*-value of < 0.05 was considered significant.

RESULTS

Burden and Demographic Characteristics

The demographic characteristics of the enrolled children in the present surveillance have been summarized in table 1. Among 238 screened fecal samples, 26 (10.9%; CI: 6.9 to 14.8) samples were Adenovirus Ag ELISA positive which showed 100% concordance with PCR test. The age distribution among children suffering from adenovirus infection is shown in table 1. High incidence of adenovirus infection was observed among ≤12 months of age (16.3%) as compared to the other age groups (*P*=0.17). Table 1 shows frequency of enteric adenovirus infection was higher in females (15.1%) compared to males (8.3%) however statistically not significant (p=0.1). Enteric adenovirus infection is highest in the children with severe dehydration (33%),followed by children with some dehydration (11.2%) and none of the children have enteric adenovirus infection without any dehydration(Table 1).Adenovirus prevalence was higher (14.9%) in children with episodes of diarrhea ≥ 10 times compared to children with ≤9 times(*P*= 0.047). Moreover, restlessness (50%) among the enteric adenovirus infected children are significantly higher (*P*=0.002). Other baseline characteristics including clinical presentations are shown in table 1.

Table 1: Demographic/clinical characteristics of the children enrolled in the present study.

Demographic and clinical characteristic		Total enrolled cases N=238	Enteric Adenovirus positive N= 26	*P value*	OR	CI 95%
Sex	Male	145	12 (8.3%)	0.1	1.0 (ref)	
	Female	93	14 (15.1%)		1.9	0.86 - 4.4
Age in months	0 – 12	49	8 (16.3%)	0.17	1.0 (ref).	
	13 - 24	76	6 (7.9%)		0.43	0.14 - 1.3
	≥ 25	113	12 (10.6%)		0.6	0.23 - 1.6
Diarrhea	≤ 3 days (duration)	121	11 (9.1%)	0.35	1.0 (ref)	
	≥ 4 days (duration)	117	15 (12.8%)		1.5	0.64 to 3.3
	≤ 9 times (episodes)	117	8 (6.8%)	0.047	1.0 (ref)	
	≥ 10 times(episodes)	121	18 (14.9%)		2.4	1.01-5.7
Vomiting	No vomiting	66	7 (10.6%)	0.27	1.0 (ref)	
	≤ 2 days (duration)	126	11 (8.7%)		0.8	0.3 to 2.2
	≥ 3 days (duration)	46	8(17.4%)		1.8	0.6 to 5.3
	≤ 3 times (episodes)	90	9 (10%)	0.9	0.9	0.33 to 2.6
	≥ 4 times (episodes)	83	10 (12%)		1.15	0.41 to 3.2
Hydration	No dehydration	11	0 (0%)	0.23	-	-
	Some dehydration	124	25 (11.2%)		1.0 (ref)	
	Severe dehydration	3	1 (33.3%)		2.0	0.2 to 22.7
No lethargy		120	11 (9.2%)	0.38	1.0 (ref)	
Lethargy		118	15 (12.7%)		1.4	0.6 to 3.3
No restlessness		232	23 (9.9%)	0.002	1.0 (ref)	
Restlessness		6	3 (50%)		9.1	1.7 to 47.6
Feeding well		125	11 (8.8%)	0.27	1.0 (ref)	
Not feeding well		113	15 (13.3%)		1.6	0.7 to 3.6
Temperature*> 37°C		125	12 (9.6%)	0.58	1.0 (ref)	
Temperature* ≤ 37°C		110	13 (11.8%)		1.3	0.55 to 2.9

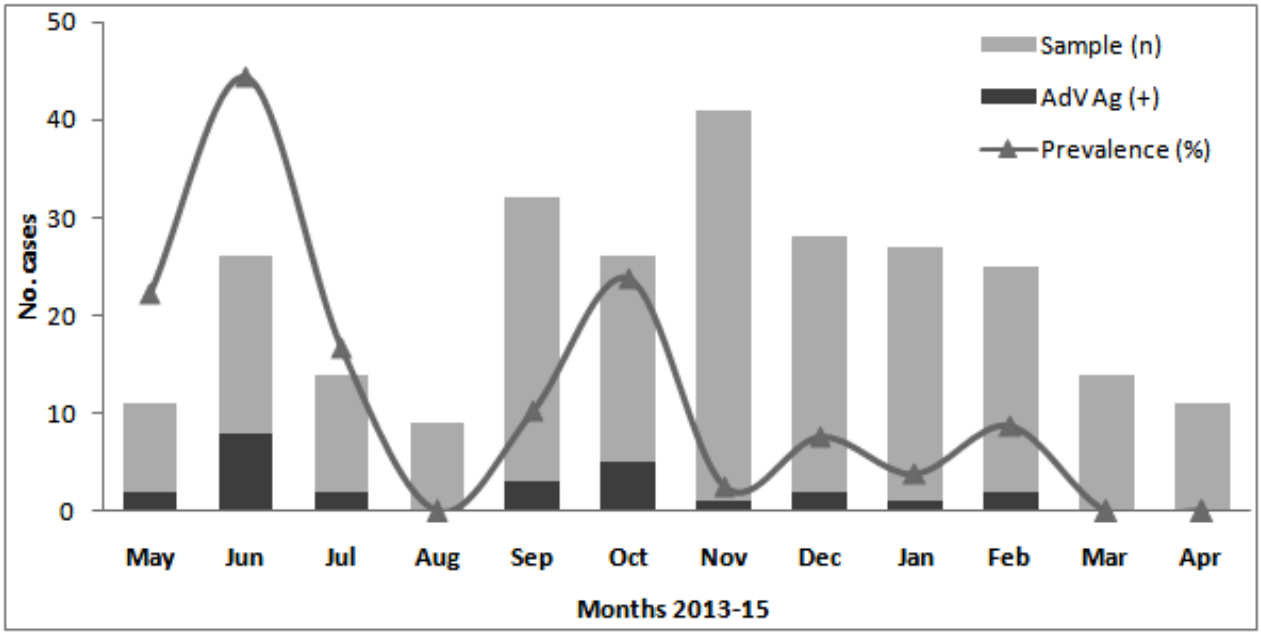

Fig 1: Epidemic curve shows prevalence of enteric adenovirus infection in children hospitalised for AGE in AMCH, Dibrugarh, Northeast India during 2013-15.

Infection Seasonality

To determine the role of seasonal changes in the prevalence of enteric adenovirus, data was analyzed on a monthly basis for 2 years (2013-2015). Prevalence of enteric adenovirus infection was higher during May to July and in the month of October, though sporadic cases wereobserved throughout the year (Fig 1).

Additionally, the months of the study were divided into the season of the year i.e. warmer months (April through September) and cooler months (October through March). Frequency of enteric adenovirus infection was higher during warmer months (17%) than cooler months (7.3%) (*P*=0.0001).

Phylogenetic Analysis

Molecular evolutionary analysis on the hexon gene showed type 41 to be the prevalent genotype in the study population. Type 40 was detected in only one sample. The hexon gene type 41 sequences from the study region created a single genomic type cluster in the population (Fig 2). All the six (type 41) sequences had a very high degree (100%) of similarity with those of the Thailand (KC632630) and South Korea (HQ326162) rather than from other sequences available from rest of India. The only hexon gene type 40 sequence from the present study had very high degree (100%) of similarity with the available sequences from Thailand (Fig 2). The average mean distance of hexon gene from the study region (Dibrugarh) for type 41 strains is 0.1%; between Dibrugarh type 41 and 40 strains is 13.2%; between Dibrugarh type 41/40 and rest of the strain is 0.7%/0.4%; between Dibrugarh type 41/40 and out group is 24.8% & 25.6% respectively.

Molecular evolutionary analysis based on the nucleotide sequences of the shaft region of the fiber gene revealed presence of two sub-lineages (DIB-1 and DIB-2) in the study population (Fig 2). Out of 12 sequences from the present study, five each clustered into the DIB-1 and DIB-2. Two sequences does not cluster into any of the two sub lineages and the sequences had a very high degree of similarity with those of the two Kolkata strains (HQ010343, HQ010350) (Fig 2). The average mean distance of shaft region of fiber gene within the Dibrugarh type 41 strains is 0.3%; between Dibrugarh type 41 and prototype TAK strain (X16583) is 1.8%; between Dibrugarh type 41 DIB-1 and DIB-2 strain is 0.5%; between Dibrugarh type 41 DIB-1/DIB-2 and distance between the two strains which did not fall in any of the two sub lineages is 0.3%. For the fiber gene the average mean distance between the Dibrugarh type 41 and type 40 is 8%.

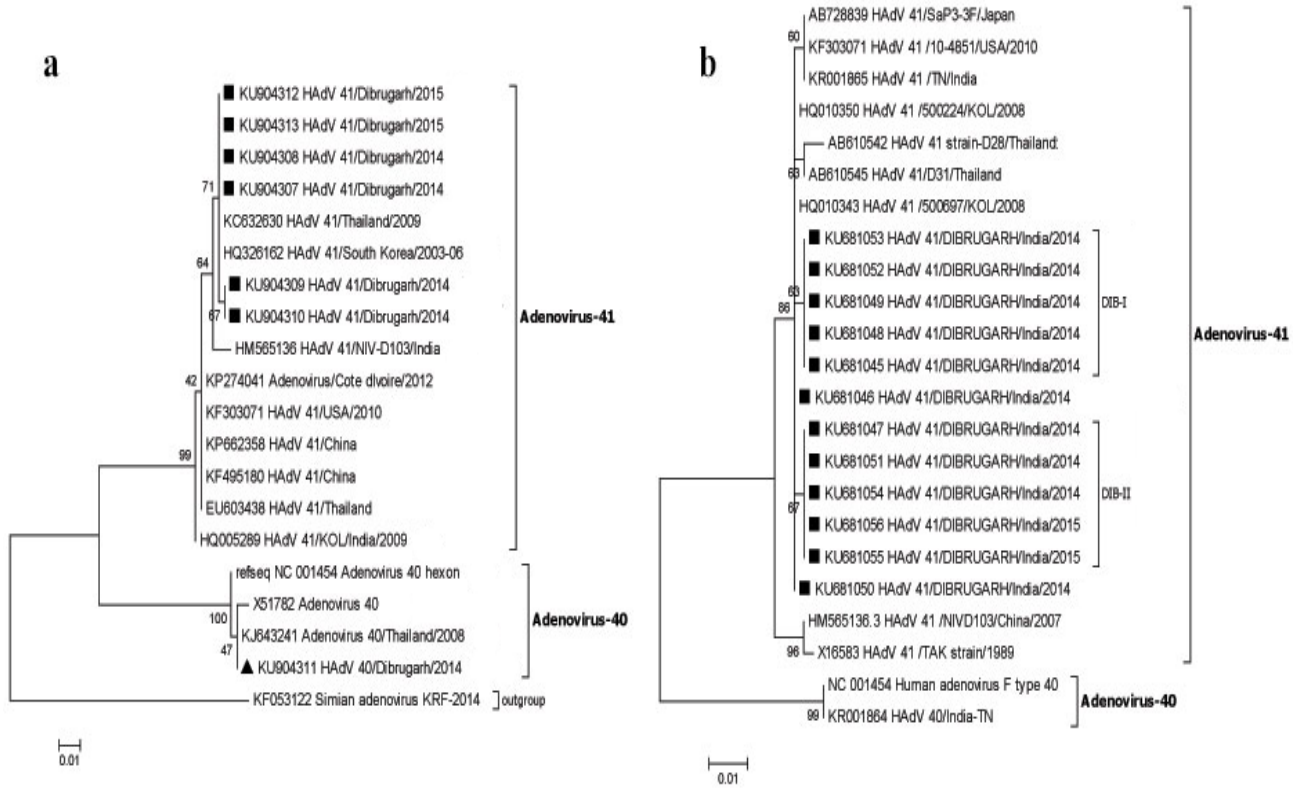

Fig 2: Phylogenetic tree based on the nucleotide sequence of the hexon gene (a) and the shaft region of the fiber gene (b) of representative adenovirus strains from children in Assam, northeast India, during May 2013 through April 2015. The phylogenetic trees were constructed by the neighbor-joining method with 1,000 bootstrap replications in the Clustal W program. The numbers at internal nodes indicate the bootstrap values. The designations indicate the strain accesion no./type/place of origine/year of infection for the strains detected in this study. DIB-I and DIB-II denote sub-leneage clusters in the fiber gene. For the hexon gene (a) and the shaft region of the fiber gene (b) respective 13 and 11 nearest respective related sequences and reference/prototype sequences from GenBank. Simian adenovirus (KF053122) strain is used as outgroup for hexon gene analysis.

Deduced Amino Acid Analysis

In the present study, clustalW analysis of all the deduced amino acid of the adenovirus shaft region of fiber gene sequences from Dibrugarh showed 15 amino acid deletions against the prototype TAK strain (Fig 3). This deletion of the 15 amino acids was confined to 15th repeat motif in the fiber gene shaft region. Additionally, two substitutions were also observed in the 199th and 250th position i.e. serine (S) is substituted by asparagine (N) and valine (V) is substituted by phenylalanine (Fig 3) Among the 15 partial adenovirus-41 hexon protein strains analyzed, there was no dissimilarity observed at the amino acid sequences. Similarly, no mutation was observed at the amino acid sequences of the partial adenovirus-40 hexon protein also.

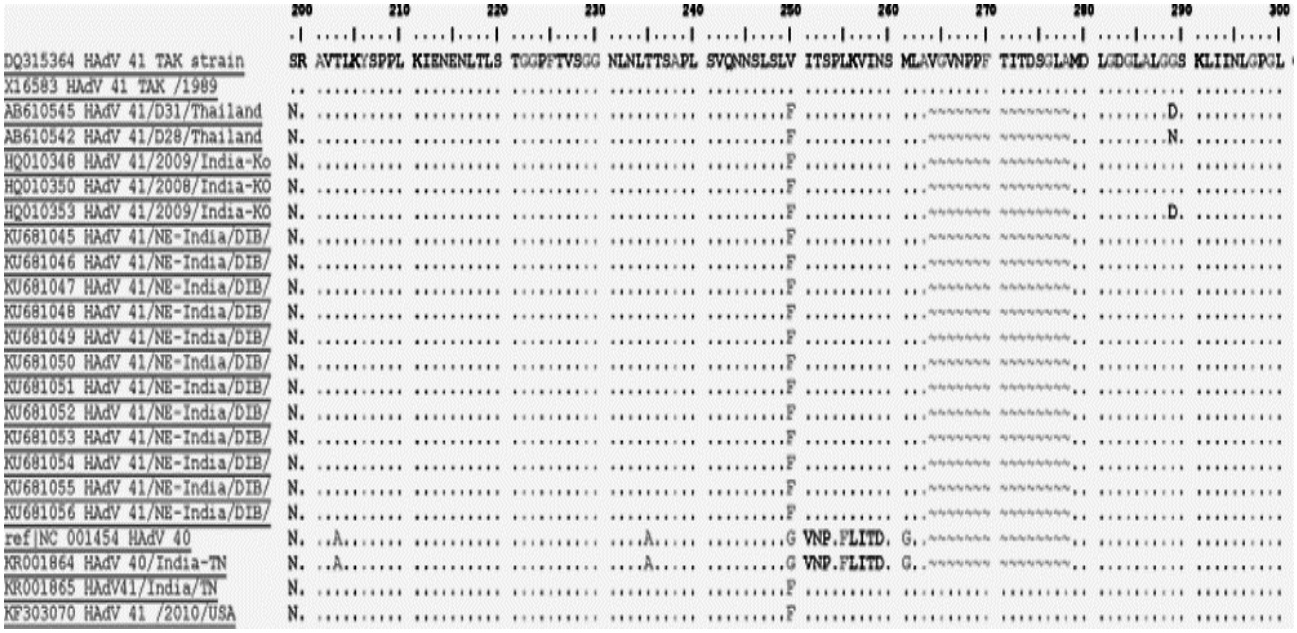

Fig 3: Alignment of the deduced amino acid sequences of the shaft region of fiber gene from 12 adenovirus type 41 strains from the present study.

DISCUSSION

Viral gastroenteritis is one of the major health problems that cause considerable disease burden in both developed and developing countries.[19] Even though rotavirus is the major viral pathogen responsible for AGE, however enteric adenovirus has been reported to cause sporadic infections and outbreaks of gastroenteritis.[8,20,21] The molecular epidemiology of enteric adenovirus species from northeast India is described for the first time in the present study. The study highlights the prevalence and seasonality of enteric adenovirus among the hospitalized children with AGE in Dibrugarh, Assam.

Epidemiological studies of enteric adenovirus in infants and children with AGE have shown its importance in other parts of India as well as globally. Prevalence of enteric adenovirus reported from western India, Pune, Aurangabad, and Nagpur showed respectively 9%, 7%, and 7.5% and from eastern India, Kolkata showed 6.5%.[22,23] Enteric adenovirus prevalence reported from other parts of the world, Alabania, China, Japan, Iran, Bangladesh and Tanzania showed 9.8%, 9.8%, 8%, 6.5%, 2.8% and 1.8% respectively.[8,11,24-27]

Enteric adenovirus diarrhea was prevalent sporadically during the study period 2013-15 and concurred with the recent reports from rest of India.[22,23] In the current study the enteric adenovirus prevalence (10.9%) in diarrheal stool specimens from Dibrugarh was much higher than that previously reported from other Indian regions as well as other parts of the world.[22,23] These findings revealed the implication of enteric adenovirus burden in this region. Globally, the patterns of diarrheal etiology are changing, with viral pathogens becoming more frequent.[28] This may be rather due to improved diagnosis for viral pathogens in recent years. Moreover, in the present investigation it was observed that adenovirus is more prevalent during warmer months contrary to rotavirus diarrhea which is more prevalent in the cooler months also known as winter diarrhea, so we may call adenovirus diarrhea as summer diarrhea.

It has been generally thought that enteric diarrhea caused by adenovirus is milder than rotavirus diarrhea.[29] However, clinical features described here showed severe diarrhea, (≥10 times) and restlessness is significantly higher in enteric adenovirus infected children. Moreover, enteric adenovirus infection in children <12 months and females is higher compared to children >12 months and males respectively. Of the clinical features that were examined, among the adenovirus infected children (26/238) frequency of dehydration (100%), lethargy (58%) and not feeding well (58%) are alarmingly higher. These findings reveal that enteric adenovirus can also cause severity similar to rotavirus.

The hexon gene and shaft region of fiber genes are considered prone site for base mutations and recombination, which were responsible for adenovirus serotype evolution.[30-33] The hexon protein and fiber protein collectively enables the virus to attach to the cellular receptor and plays important role for the serotype specificity of adenovirus strains. Genotyping was done for both the hyper variable regions of the hexon gene and shaft region of fiber gene, so that genetic variation between the strains does not get overlooked.[34] In general, the fiber gene exhibited greater genetic variability than the hexon gene. The phylogenetic analysis confirms, the hexon gene clustered into one lineage; and the fiber gene clustered into two sub lineages DIB-1 and DIB-2.

Amino acid sequences analysis of the fiber adenovirus 41 gene from the present study, showed deletion of 15 amino acids. There were 22 and 21 repeat motifs in the shaft region of the adenovirus-41 and adenovirus-40 fiber polypeptide respectively. The coding region of the shaft region was intact, as the entire 15 aminoacid coding region from both adenovirus-40/41 had been excised from the sequence. This deletion of the 15th repeat motif from the circulating adenovirus-41 strains reduced the sequence size of fiber gene, and became same size as the fiber adenovirus-40 gene. It seems that adenovirus-40 and this adenovirus-41 strain circulating in Assam, northeast India, apply similar mechanism to interact with the host receptor. In the circulating strains from Dibrugarh, this deletion of 15 amino acids is evolutionarily conserved. This diversity of AdV-41 has been reported earlier also, from where strains AB610545

Adenovirus41/D31/Thailand, HQ010348 Adenovirus41/2009/India-Kolkata, Ad41/D6/17951/Netherlandsand Ad41/D8/N7761/Canada had similar deletions. However, the gene sequences from Netherlands and Canada are not available in GenBank for amino acid sequences analysis against the enteric adenovirus strains from Dibrugarh. The shaft region of fiber has a triple β-spiral motif which is stable yet flexible. It has been speculated that a shortened fiber gene size may facilitate rapid replication and/or may have association with pathogenicity; however no concrete evidence has been reported yet.[35]

In conclusion, the present investigation reveals existence of enteric adenovirus-40/41 strain in hospitalized children with AGE and is an important pathogen in causing AGE among children aged below 5 years in northeast India. This is the first report on molecular characterization of enteric adenovirus-40/41 strains, revealing the presence of unique 15 amino acids deletion in the shaft region of the fiber gene from this region, similar to the study conducted at Kolkata, eastern India.[23] Moreover, for the first time investigation of the seasonality of enteric adenovirus infection from this region, revealed sporadic incidences throughout the year and comparatively higher incidences in the month of May to July and October.

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