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PHYLOGENETIC ANALYSIS OF VP7 GENE OF ROTAVIRUS STRAINS CIRCULATING IN CHILDREN WITH ACUTE GASTROENTERITIS IN CHENNAI WITH FOCUS ON G2

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Abstract:

Background: Group A rotavirus is the leading cause of gastroenteritis among children worldwide and accounts for 872000 hospitalizations, 3270000 outpatient visits and predictable 78000 deaths annually in India. As the disease burden is more common in low income countries including India, monitoring and detection of the circulating strains of rotavirus needs to be monitored. This study emphasis the genetic relatedness among G2 rotavirus strains that was analyzed during the study period in and around Chennai, Tamilnadu during pre-vaccination era.

Materials and Methods: We collected fresh stool from child < 5 years admitted with acute gastroenteritis (AGE) admitted to hospitals. Rotavirus infections were detected by enzyme immune assay. Positive rotavirus isolates were genotyped using semi-nested type-specific multiplex PCR for G and P types and were sequenced. Phylogenetic analysis of VP7 genes of G2 were carried out and the variations between strains isolated globally were documented.

Results: Group A rotaviruses was detected in 77 (39.48%) samples and samples of G2 was identified in more percentage and results indicated it would be re-emerging strain/genotype. The deduced amino acid sequences of the antigenic regions of VP7 exposed substitutions at various positions correlated with changes compared to other strains by phylogenetic analysis.

Conclusion: Since rotavirus is transmitted through oral-fecal route and monitoring of environmental cleanliness is of mandatory to cease the spread of this deadliest viral agent and covering single genotype/strains before the pre-vaccination era will have to be modified with respect to the circulating strains as vaccine candidate.

Keywords: Genetic Diversity, Genotypes, Phylogenetic Analysis, Severity of Diarrhea, RT-PCR, G2.

Introduction

Rotaviruses are triple-layered units of the Reoviridae family that comprise 11 segments of double-stranded RNA. Outer-layer is poised of VP7 and VP4 proteins, encoded by gene segments 9, 7, or 8 (that depends on the strain) and 4, respectively. The outer capsid is made of two proteins, VP4, also named “P protein”, and VP7, also known as the “G protein”, which define the “P” and “G” serotypes of the virus, respectively. Both are key neutralization determinants on the surface of the virion. The inner capsid is made of the VP6 protein, the most abundant and immunogenic protein in the virion. NSP4 is a viral enterotoxin which induces diarrhea and was the first viral enterotoxin discovered. [1]

Rotavirus enteritis is generally an easily controllable disease of childhood, but in 2013, 37 percent of deaths due to diarrhea and 215000 deaths worldwide has been caused by rotavirus. [2] Most of the deaths occurred in developing countries [3] and incidence and severity of rotavirus infection has declined significantly in developed and countries that have included rotavirus vaccine to their routine immunization schedule programs. [4, 5]

However, the results of many studies have been incomplete due to the limited availability of MAbs specific for types other than G1 to G4, the relatively low sensitivity of the method due mainly to the requirement of intact virus particles, or to the existence of monotypes or antibody escape mutants within the different G types. Monotypes within G1, G2, G3, and G4 rotaviruses react with different degrees of affinity against different panels of G-specific MAbs. [6]

Early microbial exposure and pediatric malnutrition result in an estimated 2.4 to 3.3 million childhood deaths due to diarrhea per year in developing countries. Efforts to improve sanitation and provide clean water have not decreased the high mortality due to rotavirus infection in developing countries and preventing infection within the first 2 years of life, when rotavirus disease is most life threatening. [7] Rotavirus immunization amid the Asian population would forestall about 110,000 deaths, 1.4 million hospitalizations and 7.7 million hospital outpatient visits. In fitting together to this study, there are two important questions: (i) will the G2 strain DS-1, isolated in 1976 and included in bovine-human reassortants, confer enough homotypic protection to protect against the G2 strains currently in circulation and (ii) will a monovalent

vaccine elicit enough of a heterotypic response to even protect against G2 strains

Rotavirus has an incubation period of 1-3 days and the gastrointestinal disorders usually resolves within three days if treated and the temperature of patients were recorded to be >39˚C. There has been considerable year-to-year variation among the circulating G-P types, wherein G1P[8] was predominant in 1998 (42% of samples) and in 2002 (26%), G2P[4] was the strain that was most frequently detected in 2000 (26% of samples). [1] There are at least 32 types of G and 47 types of P serotypes of human rotavirus where G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] preponderates the infections amid humans. [8]

The aim of the present study was to analyze the circulation and commonness of human rotavirus genotypes in and around Chennai among hospitalized children under five years of age owing to gastroenteritis with focus to reemerging G2 strains.

Materials and Methods

Sample collection

Stool samples has been collected from 195 children less than five years of age admitted to hospitals due to acute gastroenteritis as per case definition by WHO, during January to December

2015. This study was approved by Institutional ethical committee.

Children who developed diarrhea and dysentery after admission or more than 14 days of symptoms and neonates were excluded from this study. To our knowledge we came to know that the children admitted were not exposed to vaccination for rotavirus during the study period The stool samples were diluted with distilled water to 10% suspensions and clarified by centrifugation at 10,000g for 10 min. The supernatant were collected and stored at 30°C until used for the detection of group A rotavirus.

Sample processing

A 10% (v/v) suspension of antigen-positive stool samples was prepared in phosphate- buffered saline (PBS). The fecal suspension was vortexed and centrifuged at 3500g for 15 min at

4°c. By using QIA amp viral RNA Mini Kit, Qiagen GmbH, and Hilden, Germany, - 140µl of the supernatant was then used for RNA extraction according to manufacturer's instructions. Genotyping

Extraction of RNA from samples that tested positive for rotaviral antigen were subjected to RT-PCR for detecting VP6 using viral RNA kit (QIAgen Gmbh Hildem,Germany) which yielded a PCR product of 336bp. Before addition of RNA to RT-PCR mix, sample RNA was subjected to denaturation at 95°c for minutes followed by immediate incubation in ice for 3 minutes to separate double strand and this single strand was used as template for RT-PCR to amplify VP6 gene using Invitrogen one step RT-PCR kit.

G/P genotyping

Those that were positive for VP6 were subjected to genotyping using semi-nested primers for RT-PCR to amplify VP4 and VP7 for G and P genotyping using multiplex PCR primers as described. In the first round PCR 881bp region of VP7 gene was identified using VP7-F and

VP7-R primers, then the first round product was used for second round along with mixture of

VP-7 F and specific reverse primers towards G1,G2,G3,G4,G8,G9 G10 and G12 genotypes

[20]. For P genotyping a region of 876bp of VP4 was amplified using con2 and con3 primers and first round was used as template for second round where con3 was used as VP4-F and mixture of P[4], P[6], P[8], P[9], P[10] and P[11] were used as VP4-R primers. [1]

Phylogenetic Analysis

The VP7 gene sequences of the human G2 rotavirus strain were downloaded from sequence database NCBI (www.ncbi.nlm.nih.gov) and evaluated. All the sequences were assembled by use of the MEGA (version 7) program [9] and multiple-sequence alignment was conducted with the ClustalW program for the major coding regions of the VP7 gene for Human rotavirus isolates. Phylogenetic tree was constructed by using a Neighbour-joining and bootstrap analysis (n _ 1,000) program to determine the best fits for the VP7 genes. Major branches with bootstrap values of

>45% were identified as distinct groups. The amino acid sequences of the selected VP7 genes were downloaded from the protein sequence database of NCBI and the multiple-sequence alignment was conducted by ClustalW program to identify the mutations in different positions by comparing the protein sequences with the corresponding sequence.

G2 along with P[4] and other strains of rotavirus nucleotide sequence isolated from Chennai and surrounding population has been submitted to GenBank and obtained accession numbers from KY774435 to KY774443.

Results

During the study period we detected 77 cases out of 195 (39.48%) was found to be positive for rotavirus by antigen and multiplex PCR detection. It was noticed that G2P[4]

(18.1%), followed by G9P[4] (17%), G4P[4], G1P[8], G3P[8], G1P[6], G9P[9] and G9, G1 with P untypables and P[4] with G untypables and unusual strain G12P[6] and G2 with P untypables which we found to be re-emerging strain in this study period.

Phylogenetic analysis for genetic relatedness (Figure1) has been done and the results from multiple sequence alignment shows that our current isolated G2 strain has multiple mutation in its amino acid residue of the antigenic regions B (amino acids 102 and 115) and E (amino acids 258-261) where threonine is replaced by alanine and valine by isoleucine and valine replaced by phenylalanine, glutamine by lysine, arginine by glutamic acid and leucine by isoleucine. (Figure 2 and 2a)

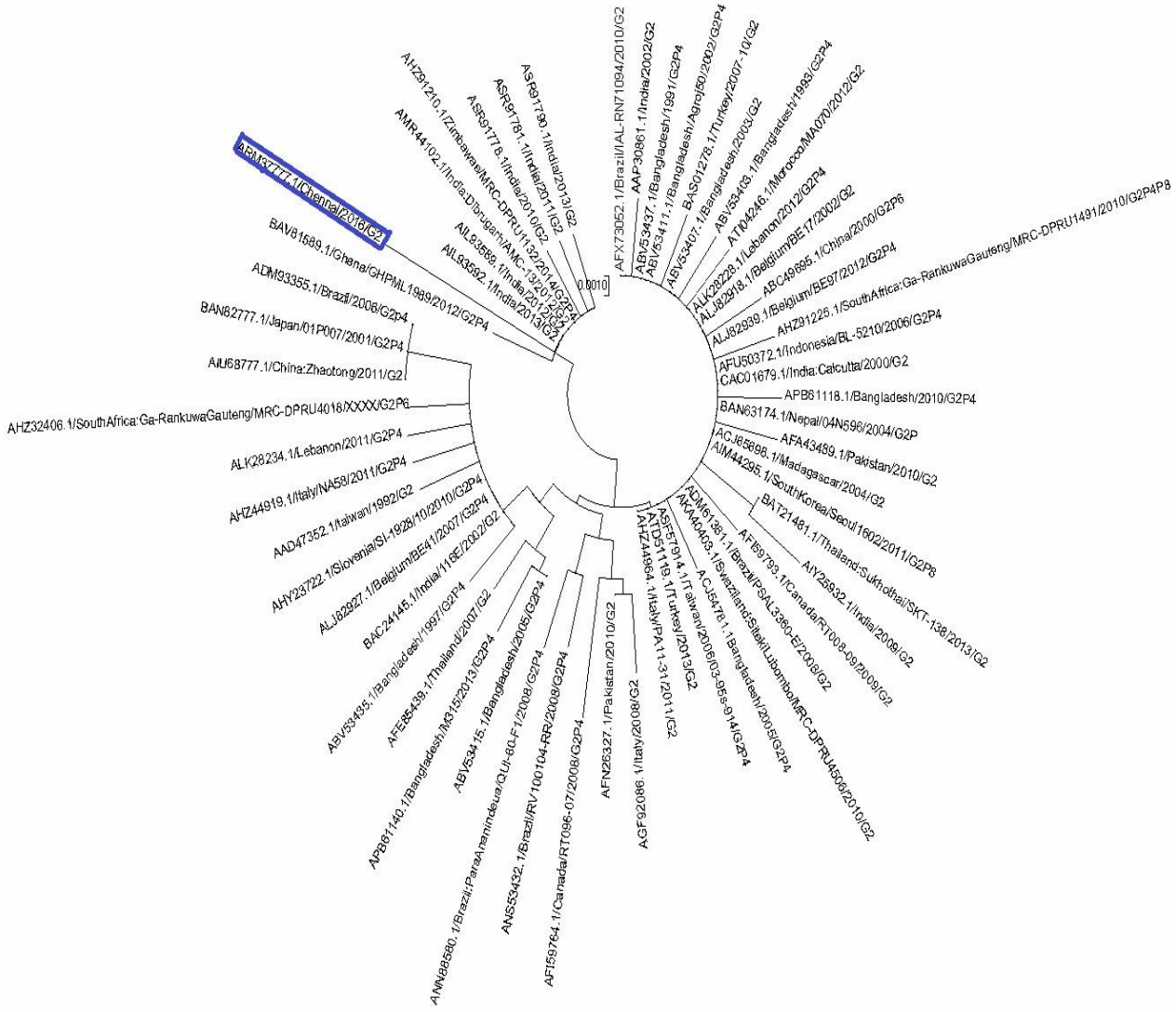

Figure 1:

Phylogenetic analysis of the VP7 deduced amino acid sequences of African serotype G2 strains.The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.13921765 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [2] and are in the units of the number of amino acid substitutions per site. The analysis involved 57 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 261 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [3]

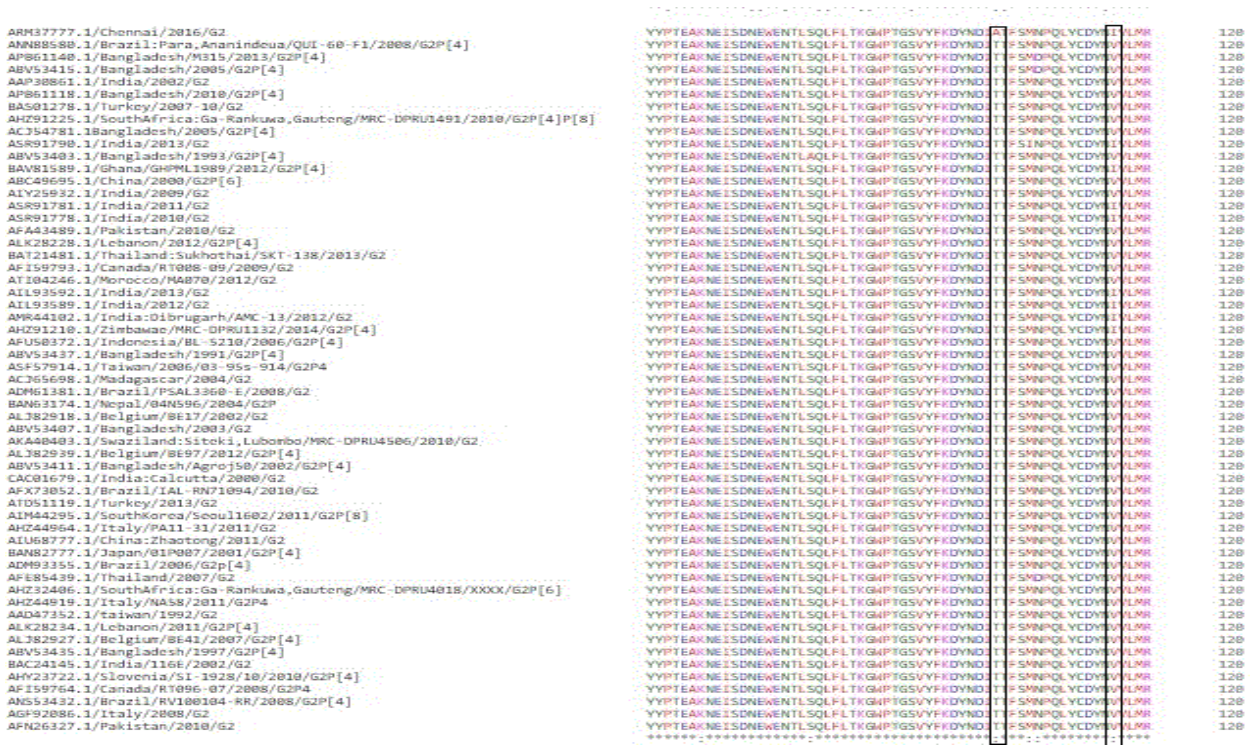

Figure 2:

Amino acid sequences of antigenic regions B (positions 102 and 115) of the VP7 genes of Chennai G2 strains.

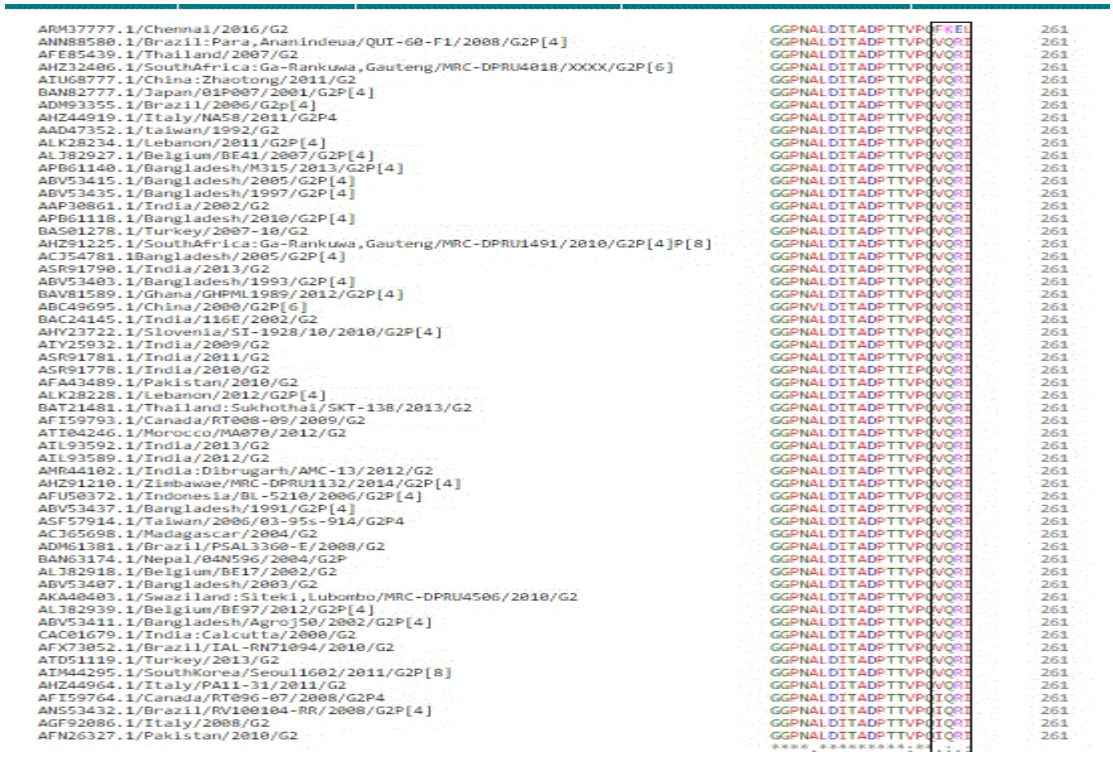

Figure 3:

Amino acid sequences of antigenic regions at E position (258 to 261) of the VP7 genes of Chennai G2 strains.

Discussion

RVV implementation has lagged in Asia compared with other regions before few years. Here, we highlight the evolving genotypes and, the circulation and prevalence of human rotavirus genotypes in and around Chennai among hospitalized children under five years of age due to gastroenteritis. Tate et.al., states that there are five countries that accounts for more than half of all diarrheal deaths (Democratic Republic of the Congo, Ethiopia, India, Nigeria, and Pakistan) among which India alone accounts for 22% of diarrheal deaths. [10]

The objective of this study was to investigate the antigenic diversity of circulating serotype with focus to G2 strains in and around Chennai, Tamilnadu. The data will be important to determine whether serotype (G2 monotypes) may become problematic in successful vaccine development and to ensure the design of an efficacious rotavirus vaccine candidate for use in Indian children.

G2 strains often are found combined with serotype P[4]1B. The G and P neutralization antigens of serotype G2 strains may allow G2 strains to seepage immunity induced by the more common G1, G3, and G4 strains. In addition, G2 has been concomitant with more severe dehydration during diarrheal episodes in children than other common strains. The diligence of immunity to rotavirus and the virulence of G2 strains needs to be addressed. [11]

In Chennai there is heavy rainfall and reaches its highest level during October to December each year, resulting in flood of the surrounding areas and increasing the chance of fecal contamination of water. An Bangladeshi study reports of G2P[4] strain to be predominant during 2005-2006 (43.2%) and we in our study found G2 strains to be at higher percentage followed by G9 and other strains. [12]

A study from western India, Pune where they characterized rotavirus infections among the vaccinated and non-vaccinated for hospitalized children they found around 8.5% of G2 serotypes and in our study we found 11.7% of G2 genotypes. Therefor a methodical effort would be required to monitor the rotavirus infections and genotypes in children bequeathing with rotavirus infection in this region. [13]

It was found that the G2 strain isolated from Chennai had 98% identity with the reference strain in the nucleotide and amino acid level. For this study we downloaded reference strains and we have compared them with NCBI-BLAST sequences where we found that G2 strain out grouped all the strains that were downloaded and analyzed using ClustalW multiple sequence alignment. The strains isolated from Chennai was found to be re-emerging during analysis period and the change indicates that the virus is capable of undergoing genetic reassortment.

Another study from Pune , western India indicates that there is inter-and/or intra genotypic differences in a genogroup-2 constellation of G2P[4] rotavirus strains circulating in that area during 2009-2013 where they reported of circulation of a novel reassortant bearing E6

NSP4 genotype of G2P[4]. In another study G2P[4] was detected in adult patients which showed NSP4-E6 genotype and circulation of heterogenous [G2 (lineage IIC and IID) thus insisting that transmission of intergenogroup reassortants among adolescent and adult patients with acute gastroenteritis emphasizes the need for continued surveillance of emerging and re-emerging rotavirus strains. [14,15]

Study by Babji et al., among the children admitted to diarrhea caused by rotavirus from 2005 to 2016 which is pre-vaccination period reports of G2P[4] (22.4%) as the predominant strain in initial years but started to replace by G1P[8] (50%) in later years which shows rotavirus genotypes vary in time and space where our study shows the presence of G2P[4] strains in higher percentage during the study period. [16]

Nayak et al., states that G2P[4]n strains were identified at 36.6% which was the prevalent strain isolated followed by G9P[4] during their study period of 2012-2013 in Kolkata, India where we also detected G2P[4] strain in higher ratio during our study period, and also they state that strains G2, G9 and P[4] detected in adults clustered together in the phylogenetic tree with the GARV strains identified in children less than five years of age. [17]

From October 2010 to February 2012 study conducted in Andaman and Nicobar islands showed that rotavirus serotype G2P[4] was the predominant strain followed by G1P[8],G1P[4],mixed genotypes and an unusual G9 serotype where we in our study found G2 being the re-emerging strain during the study period along with other serotypes as described. [18]

Venenzulian study reports of G3P[8],G2P[4],G9P[8], and G1P[8] were liable for 50.6%, 35.6%, 5.7%, and 1.1% of rotavirus diarrhea and one uncommon strain G8P[14] was reported where they found the predominance of G3, noteworthy proportion of G2 and reasonable circulation of G9 strains prior to vaccination implementation. [19] In our study we found few amino acid substitutions in G2 strains analyzed which is similar to results observed by Iturizia Gomara et.al., during 2001 where they analyzed G2 strains that did not answer during the VP7 analysis with help of monoclonal antibodies. [6].

There was a large-scale outbreak in Taiwanese children, during 1993 and it was found that this acute gastroenteritis is associated with G2 strains. The amino acid substitutions were found at four positions, one among them is Aspartic acid replaced by Asparagine, which coincides with our study. [20] We also encountered G2 strains which was found to be as re-emerging strain, our data would help in epidemiological surveillance that is needed to find the emerging /re-emerging genotypes that could escape the protection persuaded by vaccination.

Therefore the existing available data signposts fluctuations in G/P type combinations and also indicates that geography and season of that particular area/region plays a vital role in the emergence/re-emergence of unusual types, for reasons that are poorly understood.

Conclusion

This study exhibits that prevalence of rotavirus gastroenteritis among children of less than five years age proves it has to be monitored and controlled by vaccination in this region. Therefore the vaccine formulation strategies should focus on the genetic mutations in the structure of rotavirus A that should not lack cross-protection conferring from single vaccination dose against all strains of human A rotavirus. Also the investigators and clinicians should consider rotavirus as a possible cause of acute gastroenteritis in adult's also thus spreading communal infection.

Limitations

The current study is limited to small sample size and partial sequence analysis. Therefore more genetic analyses of complete genome sequences of strains of this region would be helpful to investigate the possible reassortment events and evolution of the recently emerging G2 strains which will reveal the strains that are spread globally and helps to ensure the successful use of vaccines and to explain the vaccine failure if it occurs. We did not compare rotavirus A vaccinated and non-vaccinated children as well as their genotype predominance pattern.

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