Report

1. Study Title: Molecular virology and epidemiology of Rotaviruses.

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Summary:

Severe gastroenteritis in children below 5 years of age is a major public health problem in humans globally. Worldwide approximately 10.6 million children die before their fifth birthday of which 20% deaths are attributed to diarrhoeal diseases. Although large number of bacterial, viral and parasitic pathogens have been implicated to cause diarrhoea, but rotavirus has been identified to cause severe diarrhoea and approximately 453,000 deaths among children <5 years of age .

Rotavirus is a eukaryotic pathogenic virus, member of Reoviridae family, contains segmented double stranded RNA (composed of 11 segments) causing infection (throughout the world) mostly among infants and children at the bowels resulting severe diarrhea. The name rotavirus comes from the characteristic wheel-like appearance of the virus when viewed by electron microscopy (the name rotavirus is derived from the Latin rota, meaning "wheel"). Based on the antigenecity of inner capsid protein VP6, rotaviruses are classified into seven groups (A-G). Among these groups, group A rotavirus (RVA) is the major viral pathogen of diarrheal diseases in infants and young children. The outer capsid layer is composed of two proteins, viral capsid glycoprotein VP7 defining G types and the protease sensitive protein VP4 defining P types. Based on nucleotide identity cut-off percentages, different genotypes were defined for each genome segment. A nomenclature for the comparison of complete rotavirus genomes was considered in which the notations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx are used for the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 encoding genes, respectively. At present, 27G, 37P, 13I, 6R, 6C, 7M, 16A, 6N, 8T, 12E and 8H genotypes of rotavirus have been confirmed by this Group (RCWG) recently.

In humans, rotaviruses of type G1-G4, and P[4] and P[8] are responsible for majority of infections. However recently, unusual G – P types like G5, G6, G8, G9, G12 and P[6] are being increasingly found in humans. Genotype G9 viruses re-emerged in mid 1990s and now represent fifth most common G type of clinical importance. Unlike other common genotypes (G1-G4), which occur commonly with P1A[8] or P1B[4], G9 viruses have been detected with a variety of P types including P[4], P[6], P[8], P[11], P[19] [19]. The segmented nature of rotavirus genome provides mechanism for generation of genetic diversity by process of genetic reassortment that occurs during mixed infections. This reassortment among co-circulating animal and human strains may contribute significantly to genetic diversity of rotaviruses and has serious implications in designing strategies for future rotaviral vaccines .

Purpose:

- To estimate the frequency of Rotavirus infection among hospitalized children and characterize the circulating genotypes
- Full genomic analysis of unusual animal-like strains identified during the study to understand their evolution.

Materials and Methods:

ELISA

The stool samples were screened for rotavirus using an enzyme-linked immunoassay (EIA) detecting the VP6 antigen as per the manufacturer's instructions (Rota IDEIA, Thermo Fisher, Waltham, MA, USA).

Viral RNA extraction and Genotyping

All the rotavirus positive samples, detected by ELISA, were confirmed for positivity by reverse transcription and PCR to avoid a false positive result. RVA double-stranded RNA was extracted from feces of positive samples by using a commercially available RNA extraction kit (QIAamp viral RNA Mini Kit, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Complementary DNA was synthesized from the extracted viral RNA through reverse transcription in the presence of random hexamers. G and P genotyping was performed using VP7- and VP4-specific multiplex semi-nested RT-PCRs as described previously [33]. PCR products were purified with a QIAquick PCR purification kit (QiagenGmbH, Hilden, Germany).

Nucleotide sequencing and phylogenetic analysis

Nucleotide sequencing was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, California, USA) in an ABI Prism 3730 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA) as described previously. Nucleotide and protein 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.

Pairwise sequence alignments were performed using LALIGN software (EMBnet, Swiss Institute of Bioinformatic, Switzerland), and multiple alignments were done with DDBJ software and CLUSTAL W. Amino acid sequences were deduced using the TRANSEQ software (Transeq Nucleotide to Protein Sequence Conversion Tool, EMBL-EBI, Cambridgeshire, UK).

Phylogenetic tree was constructed using the MEGA (Molecular Evolutionary Genetics Analysis) program, version 6. Nucleotide sequences were submitted to the GenBank database.

Results:

During the ongoing hospital based study stool samples were collected from ID-BG Hospital and B. C. Roy Memorial Hospital for Children in Kolkata. Stool samples of every fifth admitted patient (≤5 years of age) with acute watery diarrhea, vomiting and abdominal pain, were collected. The inclusion criteria for OPD patients included passing of three or more loose/watery stools within 24 h. A total of 370 stool samples were collected from hospitalized patients and 526 stool samples were collected from OPD patients. Stool samples were stored at -70°C for further study. Preliminary screening of the stool samples for the presence of RVAs was performed using ELISA as per the manufacturer's instructions. 48.8% were positive among 896 samples (Figure 1).

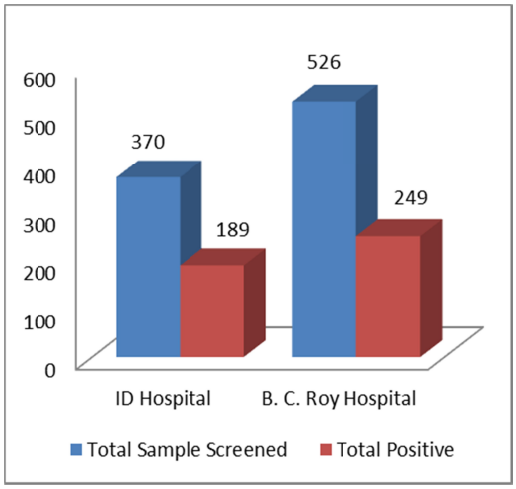

Figure 1. Samples tested by EIA and positive for rotavirus by hospitalization in 2014

The maximum number of rotavirus positivity was found in the age group of 6-12 months followed by 12-24 months of children (Figure 2) which correlates with post weaning period. No correlation between Male and female with RV positivity was observed. (Figure 3).

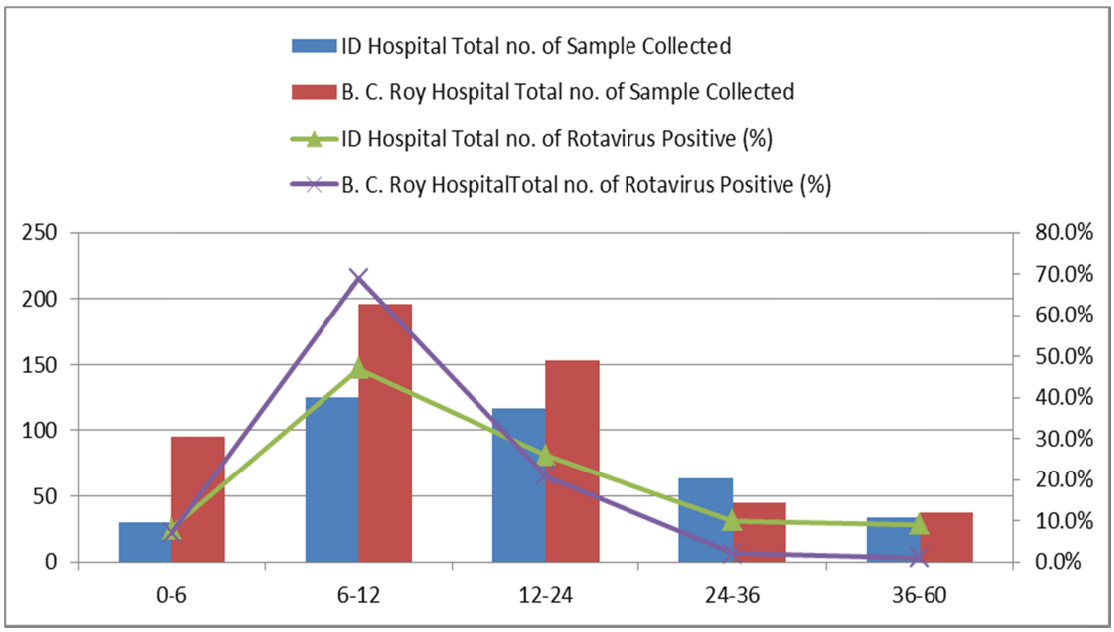

Figure 2: Age –wise distribution of collected sample and rotavirus positive samples during the year 2014.

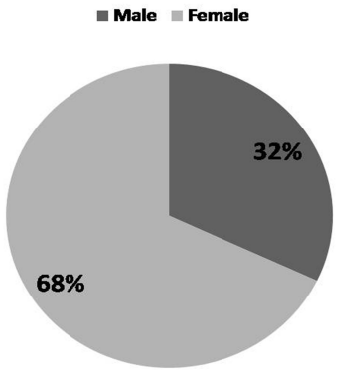

Figure 3. Male female ratio of positive samples detected during 2014.

During the year 2014 hospital based study revealed G1 strains in conjunction with P[4], P[6] and P[8] to be the most prevalent strain (54%) followed by G2 (23%) strains in combination with P[4] and P[8]. G9 strains (14%) in combination with P[4], and P[8] decreased in comparison with the previous years. G12 strains in combination with P[4], P[6] and P[8] comprised 7% of all strains. In case of P-Types P[8] was highest (54%) (Figure 4).

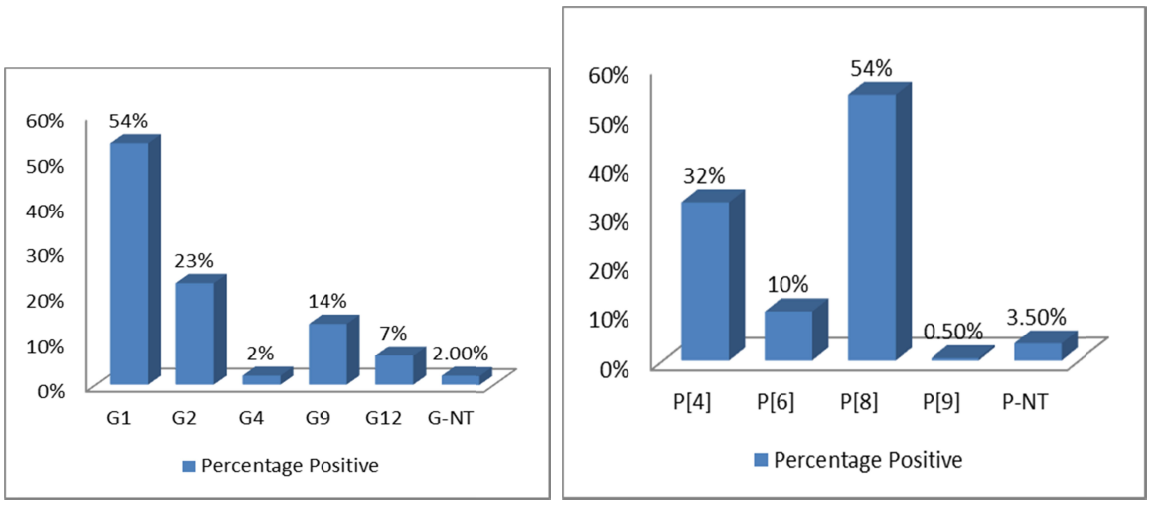

Figure 4: Percentage of G and P genotypes for the year 2014.

Analysis of uncommon strains circulating in Kolkata

A few unusual strains like G1P[9], G10P[14] and G12P[11] were detected in 2013-14 hospital based study. Full genome sequencing of uncommon strains is underway.

- **G1P[9] :** Analysis of VP7 and VP4 gene of this strain RVA/Human-wt/IND/IDK-6057/2013/G1P[9] revealed this strain has both human-like VP7 and VP4 gene. This strain was isolated in 2013 from a child with severe diarrhea. P[9] genotype is a feline-like genotype but also found in human in less number and in combination with G3 genotype.
- **G10P[14] :** Analysis of VP7 and VP4 gene of this strain RVA/Human-wt/IND/BCK-3629/2013/G10P[14] revealed this strain has bovine-like VP7 and VP4 gene. This strain was isolated from a child with mild diarrhea in 2013.

- **G12P[11] :** Analysis of VP7 and VP4 gene of this strain RVA/Human-wt/IND/BCK-3652/2013/G12P[11] revealed this strain has human-like VP7 and bovine-like VP4 gene. This strain was isolated from a child with mild diarrhea in 2013.

Table 1: Full genome characterization of uncommon strains

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-wt/IND/2013/G1P[9]	G1	P[9]	I2	R1	C2	M2	A1	N2	T1	E1	H1
RVA/Human-wt/IND/2013/G10P[14]	G10	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
RVA/Human-wt/IND/2013/G12P[11]	G12	P[11]	I1	R1	C1	M1	A5	N1	T1	E1	H1

Summary

- ≈50% of ≤5 years children admitted in hospitals or treated at the OPD with severe or mild diarrhea, in the same area were rotavirus positive.
- Rotavirus positivity was highest in winter seasons and children of 6-24 months age group were most vulnerable for infection.
- Strain diversity of rotavirus was higher in Kolkata. Multiple types of strains were co-circulating in this region.
- Common strains like G1P[8], G2P[4], G9P[4]/P[8] and G12P[4]/P[6]/P[8] were found during study period.

- During 2014, the most prevalent genotype was G1P[8] followed by G2P[6]
- Unusual animal-like strains like G10P[14], G1P[9] and G12P[11] represented interpsecies transmission and reassortment of gene segments in co-circulating strains.

Future Plan of Work

1. Expand and continue Rotavirus surveillance in children in rural area
2. Phylogenetic analysis of circulating Genotypes and theit correlation with vaccine strains
3. Full genome sequencing for uncommon strains